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AN ANALYSIS OF THE ACTION OF ACETYLCHOLINE ON HEARTS, PARTICULARLY IN ARTHROPODS

C. LADD PROSSER

(From the University of Illinois, Urbana, and the Marine Biological Laboratory, Woods Hole)

Two organic compounds have been shown to be liberated as mediators at nerve endings, adrenin-like sympathin and acetylcholine. The distribution of these substances throughout the animal kingdom is nearly universal. Adrenalin seems to be excitatory on all adult hearts although many molluscan hearts are relatively insensitive to it. Acetylcholine has an inhibitory action on the systemic hearts of adult vertebrates and of molluscs; acetylcholine has no action on the hearts of early Fundulus embryos; it has an accelerating action on the hearts of decapod Crustacea, of Limulus, the grasshopper Melanoplus, and of the annelids Arenicola and Lumbricus; (see Table II for references). It might be possible to explain these three types of action of acetylcholine if a wide variety of hearts was compared with respect to drug effects and histological characteristics. Baylor (1942) has recently found acetylcholine to inhibit the Daphnia heart. This indicates that acceleration is not characteristic of all arthropods and it becomes important to know which groups show acceleration, which inhibition, and which no effect of acetylcholine.

In establishing the physiological action of a drug the effects must be reversible and a threshold concentration must be demonstrable. These two criteria have been adhered to in the following experiments. The mere fact that acetylcholine acts in a given manner on an effector does not imply that the nerves supplying that effector liberate acetylcholine. Mediation by a substance can be proved only when that substance is obtained in a perfusate and when potentiating and inhibiting drugs are combined with nerve stimulation. The following experiments are concerned only with drug effects, not with the liberation of substances at nerve endings.

Penetration of Acetylcholine into Intact Animals

The effects of acetylcholine upon higher arthropods have been examined by direct perfusion of isolated hearts. In working with the smaller transparent crustacea the only feasible method of applying the drug is to immerse the animals in a solution of it. To test the validity of this method a number of experiments have been performed on crayfish. Davenport, Loomis and Opler (1940) showed by perfusion that acetylcholine (10^{-10}) accelerates, raises the tone, and may stop in systole the heart of *Astacus trowbridgei*. We find the hearts of *Cambarus virilis* and *C. propinquus* to be accelerated likewise by perfusion in low concentrations. When acetylcholine is dripped over the outside of a heart which is left in situ the threshold is much higher (10^{-6} to 10^{-5}) than when perfused.

To learn whether acetylcholine would penetrate in appreciable amounts into intact animals the hearts of crayfish were exposed by removing a piece of the dorsal carapace and the animals allowed to stand in water just over the lateral margin of the carapace so that water never came in contact with the heart. The heart rates were recorded kymographically or were counted. The effect of acetylcholine applied in this way is shown in Figure 1*A*. A concentration of 10^{-4} in water accelerated in this way about as much as did 10^{-5} in saline when dripped on a heart similarly exposed. Acceleration by potassium and inhibition by calcium in the water were readily demonstrated. Eserine (physostigmine) definitely potentiated the acetylcholine acceleration. Small specimens were more sensitive than large ones, due probably to differences in dilution in the body and in the absolute amount of choline-esterase present. Maluf (1940) showed with dyes that crayfish take in very little water by mouth but that dissolved material enters by way of the gills. Since acetylcholine does not stimulate the central nervous system of the crayfish (Prosser 1940a) its action in the immersion experiments is probably directly on the heart.

It is concluded that sufficient acetylcholine enters the body of a crayfish by the gills from a solution of 1 : 10,000 to accelerate the heart by at least 20 to 25 per cent. Therefore direct application of a drug to an exposed heart is not necessary to establish its qualitative action, although thresholds by perfusion are very much lower. Penetration through the body surface (gills, etc.) has frequently been used for studying the effects of other inhibitors and accelerants on hearts, and toxic substances of polluted water must frequently penetrate animals in this way.

Effect of Acetylcholine on Non-decapod Crustacea

Amphipoda: Two species were used, *Talorchestia longicornis* and *Bactrurus mucronatus.*

Talorchestia: When a small spot of light is focused on the dorsal side of the marine amphipod Talorchestia the heartbeat can be counted very readily with the aid of a low-power dissecting microscope. The heart is very rapid and to count the beats it was slowed by cooling. Single specimens were restrained by insertion into a glass tube of slightly larger bore than their body diameter. Appropriate solutions passed through this tube. The tube was placed in a water bath which was maintained at 15 ± 0.5° C. Even at this temperature the rate is fast (about 175–200/min.) but can be counted accurately after a little practice. The counts were checked by other persons on several occasions.

Six different experiments were performed and in each of these several applications of acetylcholine were tried. The time for ten beats was counted from five to 12 times approximately every five minutes. Probable errors for individual sets of counts were 0.05 to 0.1 second, while significant differences were 0.3 to 0.5 second. The dilutions of acetylcholine were prepared by adding a stock solution (10^{-3}) to sea water. Similar dilution of sea water with distilled water (90 per cent sea water) had no effect upon the heart rate over the periods used.

Results of one typical experiment are shown in Figure 1*B*. Acetylcholine accelerates the heart of Talorchestia. Threshold by external bathing lies between 10^{-5} and 10^{-4}. There may be some eserine potentiation. Similar results were obtained on five animals.

Bactrurus: Specimens of this freshwater Amphipod were collected at the mouth of a drain from a field and were studied within a day after collection. Individual animals were placed in separate watch glasses containing test solutions. For counting, they were held under a coverslip supported at two sides by a number of coverslips held by vaseline. The number of supporting coverslips was varied according to the size of the specimen so that the abdomen was freely movable and the thorax held lightly in place. The hearts of freshly collected specimens were very steady and easily counted. Twelve experiments were performed. Acetylcholine (10^{-4}) accelerated the heart markedly (15–20 per cent) in each of eight specimens (Figure *C, D*). There was slight acceleration in two at 10^{-5} and no acceleration in two at 10^{-6} (Figure 1*D*).

It is concluded that the hearts of amphipods are accelerated as are the hearts of decapod Crustacea.

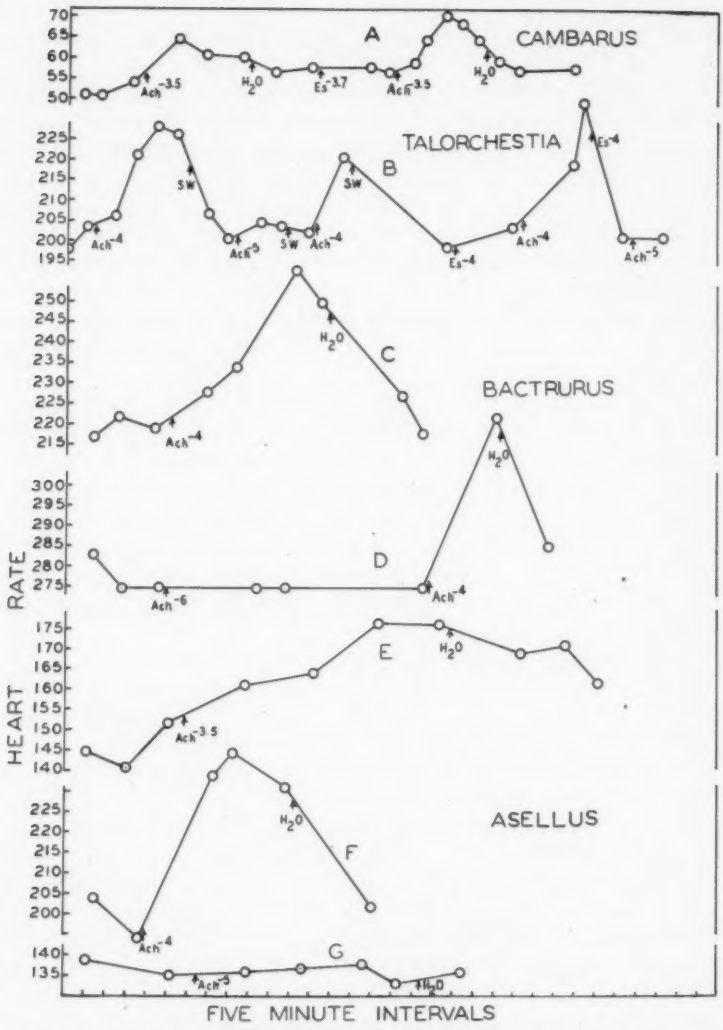

FIG. 1. Effect of acetylcholine upon the heart rate in beats per minute of (*A*) *Cambarus virilis*, (*B*) *Talorches tialongicornis*, (*C, D*) *Bactrurus mucronatus* and (*E, F, G*) *Asellus tridentatus*. Drugs applied by immersion. Concentrations given in negative exponents of 1×10.

Isopoda:

Specimens of the transparent eyeless freshwater isopod *Asellus tridentatus* were collected along with the Bactrurus.[1] Their heart rate varies more with body activity than that in any of the Crustacea which we have observed. When leg movement occurs the heart speeds up and then slows when the animal becomes quiet. In general the resting rate was taken. The animals were restrained in the same chambers as the Bactrurus and were not left on the observation slide more than five to ten minutes at a time. Freshly collected specimens had more regular hearts and were more sensitive to acetylcholine than specimens which had been in the laboratory for several days. Twenty-four experiments were performed. Acetylcholine $10^{-3.5}$ accelerated the heart in each of four specimens (Figure 1*E*). Acetylcholine 10^{-4} accelerated in eight (by approximately 20 per cent) (Figure 1*F*), and was without effect in two. In three specimens there was some acceleration with acetylcholine 10^{-5} and in four no effect (Figure 1*G*), while in three specimens there was no effect at 10^{-6}. Significant differences were three to five times probable errors. The variability of heart rate, the differences in sensitivity, and different patterns of acceleration (Figures 1*E* and 1*F*) make the preparation less satisfactory than Bactrurus but there is no doubt qualitatively that acetylcholine accelerates the heart.

Copepoda:

Diaptomus. Two species of freshwater copepod were studied, *Diaptomus oregonensis* from winter plankton and *Diaptomus sanguinensis* collected in temporary spring ponds. Nineteen experiments were performed, fifteen of these with *Diaptomus sanguinensis*. The hearts are very rapid (ten beats in less than two seconds) and the animals are too opaque to use the stroboscopic method employed by Baylor with Daphnia. Hence they were entangled in cotton placed in depression slides and these floated on a water bath held at $11 \pm 0.1°$ C. It was possible to get reproducible counts if the rate did not exceed ten beats in 1.6 seconds. The accuracy is not high but qualitatively there is little difficulty in distinguishing between a heart beating ten times in 2.0 seconds and one beating ten times in 1.6 seconds. Probable errors are of the order of 0.05 sec. and significant differences 0.2 sec. Acetylcholine acceleration was observed with recovery in pond water in each of three specimens in a concentration of $10^{-3.5}$, in each of ten specimens in 10^{-4} and slight acceleration in each of three at 10^{-5}. No acceleration was noted in two at 10^{-6}. Typical curves are shown in

[1] I am indebted to Dr. Leslie Hubricht of the Missouri Botanical Garden for identification of the specimens of Bactrurus and Asellus.

Figure 2 *H, I.* It is evident that acceleration by acetylcholine is not restricted to the malacostracan Crustacea.

Phyllopoda:

Artemia salina. Eggs of Artemia were hatched in sea water to which sea salt had been added to give a solution with a freezing point of $-2.8°$ C. The animals were fed yeast. Most of the specimens tested were adults although some were in the last or next to last instar. Attempts to mount specimens in small tubes through which test solutions passed were unsuccessful because the animals were very easily

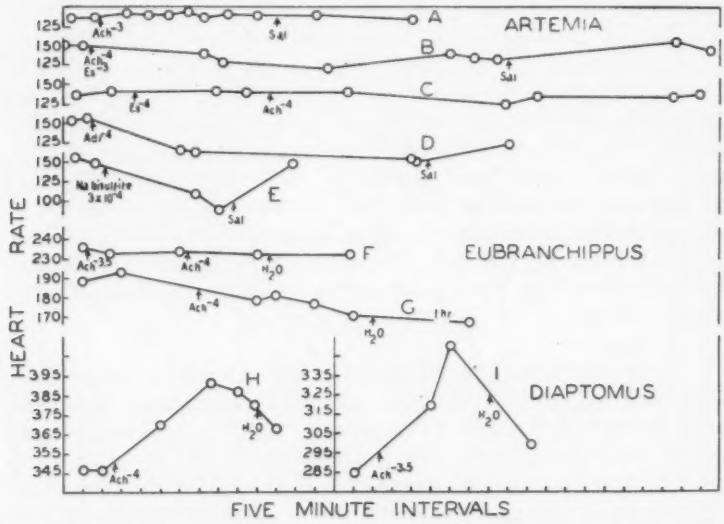

FIG. 2. Effects of drugs on the hearts of *Artemia salina* (curves *A, B, C, D, E*), *Eubranchipus serratus* (curves *F, G*) and *Diaptomus sanguinensis* (curves *H, I*). Ach, acetylcholine, Es, eserine, Adr, adrenalin. Heart rate in beats per minute.

affected by slight currents. Restraint in cotton or lens tissue was also ineffective. The best method found was to hold the specimens in a chamber supported by a variable number of coverslips as described above for Bactrurus. The specimens were placed in watch glasses containing approximately 10 ml. solution and were transferred from time to time to the restraining slides for examination. Prolonged restraint sometimes slowed the heart. Drug solutions were made up in culture fluid.

The heart was easily seen from either dorsal or lateral aspect. Sometimes a margin of the heart was observed, sometimes the move-

ment of the valvular ostia and often the rhythmic propulsion of corpuscles. The contractions of the heart occasionally seem synchronous with the movements of the gill appendages but careful count usually shows a difference in the two rhythms, the heart being slightly faster.

Acetylcholine is without effect on the heart of Artemia (Figure 2 *A*, *B*, *C*). Specimens were immersed in concentrations as high as 10^{-4} and 10^{-3} in culture fluid for periods of one to two hours. Of six animals in acetylcholine 10^{-4}, five showed no effect and one a slight slowing of heart rate. Five specimens in acetylcholine 10^{-3} showed no effect (Figure 2*A*). Eserine 10^{-4} was applied to other specimens both before and concurrently with acetylcholine. In four specimens treated with eserine for about a half hour before treatment with acetylcholine there was no alteration of heart rate by the acetylcholine. Six specimens were placed in culture fluid containing both eserine (10^{-4}) and acetylcholine (10^{-3} or 10^{-4}). In three of these there was no effect (Figure 2*C*). Three, however, showed a slight slowing of the heart (Figure 2*B*). Eserine alone applied for periods of one half hour or longer slowed in three animals and had no effect in three others. Thus the slowing in the mixture of eserine and acetylcholine may have been due to the eserine rather than to the acetylcholine. All specimens treated with eserine showed locomotor paralysis; the gill appendages often stopped beating altogether. Eserine had a much greater paralyzing effect on gill movement than on the heart. Recovery was rapid in non-eserinized culture fluid.

Adrenalin (Parke, Davis solution) was applied in concentrations ranging from 10^{-7} to 10^{-4}. Adrenalin 10^{-7} and 10^{-6} were without effect. Adrenalin 10^{-5} slowed the heart slightly and 10^{-4} slowed it by over 33 per cent (Figure 2*D*). This effect was surprising and the preservatives in the adrenalin were suspected. Chloretone in twice the concentration present in 10^{-4} adrenalin (chloretone 2×10^{-4}) was without effect. Powdered adrenal medulla was tried and there was no effect on the Artemia hearts within one half hour. However this adrenalin became partly oxidized in this time as indicated by the development of a pink color. Sodium bisulfite was added to the crystalline adrenalin solution and there was a marked slowing. When placed in sodium bisulfite 10^{-4} alone a marked slowing was observed in several specimens (Figure 2*E*). Threshold was about 10^{-5}. The apparent slowing by adrenalin is due, therefore, not to adrenalin but to the sodium bisulfite present as a reducing agent.

The lack of effect of acetylcholine and adrenalin raises the question whether these substances get to the heart. Artemia is famous for its resistance to acid fixing solutions. Krogh (1939) found that its permeability to heavy water is low but appreciable. An attempt was made to inject drug solutions. A micropipette attached by micromanipulator tubing to a syringe was used. The micropipette was inserted by hand in the thoracic region and 0.008 ml. of solution injected. Solutions of culture fluid and of acetylcholine were injected, and the pipette inserted without any injection in different individuals. The heart always stopped beating for some distance ahead of the point of insertion. Sometimes local beats would arise, particularly in the abdomen. The animals were upset and often died as the result of the mere insertion of the micropipette. It was impossible to observe any effect attributable to injected acetylcholine.

A study of penetration of dyes was then made. Animals were placed in dilute solutions of methylene blue and of neutral red. Within fifteen minutes the dyes could be seen in the intestine and in about half an hour they were noted in various tissues, even in the blood sinuses in the appendages. In contrast to the crayfish, the dyes were not taken up by the gills but were swallowed. Considerable variation was noted in the amount swallowed by different specimens. Dyes were used in the acetylcholine solution to show that the solutes definitely reached the interior of the animals. The imperviousness to fixatives may be due to the very low gill permeability and lack of swallowing in the fixative. The fact that eserine and sodium bisulfite have definite effects upon the heart and upon locomotion shows that these substances, like the dyes, enter the animal. The acetylcholine could hardly be destroyed since the medium was slightly acid and eserine had no effect. It is concluded that acetylcholine and adrenalin are without effect upon the heart of Artemia and that this lack of effect is not due to lack of penetration. It was noted that during periods of activity of thoracic appendages the heart was not accelerated over the rate at rest.

Eubranchipus:

Specimens of *Eubranchipus serratus* were collected in temporary ponds in March and April of two successive years. For observation most of the specimens were placed in small tubes through which test solutions could pass, as with the Talorchestia; a few were kept in watch glasses and mounted for observation, as were the Artemia. Essentially the same results were obtained with fourteen specimens. Acetylcholine (10^{-4} or 10^{-3}) in pond water was without effect (Figure 2*F*, *G*). Thus Eubranchipus behaves like Artemia.

Limulus embryos

It was shown by Carlson and Meek (1908) that the heart of Limulus begins beating on the twenty-first day of development, that this is before the heart ganglion is developed, that the beat continues to be myogenic until about the thirtieth day when the ganglion is first demonstrable histologically. Crozier and Stier (1927) found the temperature characteristic of the heart rate during the myogenic period to be usually $\mu = 11{,}500$ or 16,400 by contrast to $\mu = 12{,}200$ for the adult neurogenic heart. If there is a difference in the pharmacology of neurogenic and myogenic hearts one would expect the hearts of Limulus embryos to behave differently during the two periods of development. The heart of Limulus is accelerated by acetylcholine (Garrey, 1941).

Two batches of eggs were obtained at the time of laying. The several hundred eggs were kept in evaporating dishes through which sea water ran. The embryos developed during about a month and a half.

Heartbeat was first clearly visible on the twenty-first day of development. At that time about 15 embryos were mounted in numbered watch glasses so that they could be followed during the course of development. The egg capsule was removed, the chorion slit longitudinally in the mid-dorsal line so that it could be folded back at the sides of the animal. The edges of the chorion were held down by vaseline and the embryos remained permanently in this position. The watch glasses were kept in a sea water tray with water in the dishes changed at least daily. Some animals died and were replaced by others from the large evaporating dishes. Development proceeded at about the same rate in the eggs both in the watch glasses and in the large dishes. A small spot of light was focused on an embryo and observations made with a dissecting binocular. The heart rate was occasionally faster during the first one to three minutes of observation than after that time. The embryos were covered with sea water approximately 5 mm. deep and sometimes an additional water filter was used to remove any heat, but the brief initial acceleration continued. It is possible that light has a slight stimulating action on the heart. Counts were made after equilibrium was reached.

Solutions were changed in the watch glasses by pipettes and the heart rates counted (time for ten beats) from five to ten times at intervals of a few minutes for several hours. Between periods of counting, the watch glasses stood in sea water the temperature of which remained constant to within 1° C. This procedure was repeated on the same animals on alternate days for approximately two and a half weeks.

It was much easier to observe the hearts during the early period when the carapace was soft and fairly transparent. At the age of about four weeks pigment developed, the carapace hardened, and the heart could not be seen easily. It was found that in the older embryos a small area behind the median eye remained fairly translucent and light reflected from the anterior end of the heart could be observed there. After about 30 days of development the heart rates became somewhat more irregular.

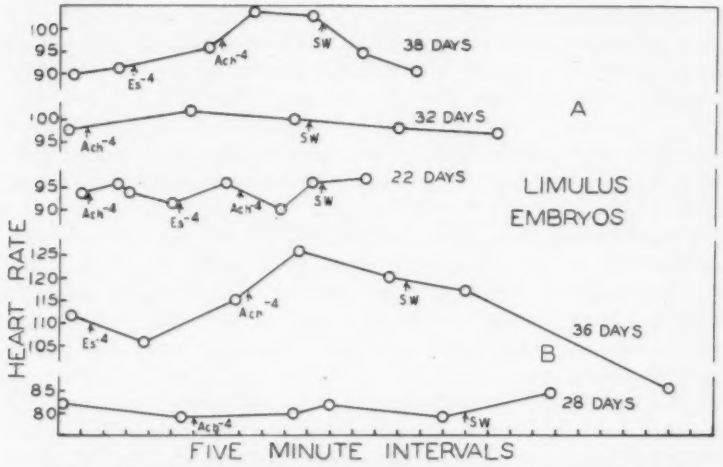

FIG. 3. Effect of acetylcholine and eserine on hearts of two Limulus embryos. Embryo *A* at 22, 32, and 38 days of development and embryo *B* at 28 and 36 days.

Data were obtained on 11 embryos. In two of these the period of observation extended over only four days but in the others data were obtained for periods between seven and 19 days. Typical curves are shown in Figure 3 and the entire experiment summarized in Table I. During the myogenic period acetylcholine had no effect upon the hearts of the Limulus embryos. Specimens were kept in concentrations of 10^{-4} for periods up to one hour. Eserine 10^{-4} was applied either with the acetylcholine or prior to treatment with it and still there was no effect of the acetylcholine. On the twenty-eighth to thirtieth days when, according to Carlson, the heart ganglion is present there was still little or no action. On the thirty-first day, however, two embryos showed acceleration and by the thirty-fifth day most of the specimens were accelerated by acetylcholine as is the adult heart. Only when the

effect was completely reversible was a positive effect recorded (Table I). It appears, therefore, that as in the Fundulus embryo (Armstrong, 1935) in Limulus a non-innervated heart is unaffected by acetylcholine.

Histological Interpretation

The preceding evidence shows (1) that acceleration by acetylcholine is not a general arthropod character, and (2) that some hearts may be accelerated, some inhibited, and others unaffected by acetylcholine. We shall seek an interpretation of the differences in acetylcholine action in terms of (1) whether the pacemaker tissue is nervous or muscular, and (2) whether there are also present regulating nerves coming to the heart from outside.[2]

Table I

Days															Embryo
22	23	24	25	26	27	28	29	30	31	32	35	36	38	40	1
0	0		0			0		0		+			+	+	1
	0		0			−		0		0	+				2
	0		0												3
				+ sl	0				+ sl						4
		0		0	0				+		+				5
						0				0	0	+			6
		0		0	0						+				7
				0	0		−		0						8
							0		0						9
								0				+ sl		+	10
										0	0	0	+	+	11

Effect of acetylcholine on hearts of embryos of Limulus from the twenty-second to fortieth days of development. 0 indicates no effect, − inhibition, + acceleration, and sl slight.

Nerve cells were first described in the heart of the crayfish by Berger in 1877 and have been seen in various decapod Crustacea by all who have looked for them since that time. Some of the more important papers are listed in Table II. Alexandrowicz (1932) has shown these cells to be large, limited in number, and to be mostly multipolar. Ganglion cells of the same sort occur in the isopod, Ligia, (Alexandrowicz, 1931). In Limulus the median cardiac ganglion contains multipolar and bipolar cells and is the pacemaker of the adult heart (Carlson, 1904). Two chains of nerve cells were described on the heart of the

[2] The hypothesis to be presented was suggested by Prosser and Zimmerman, 1941. *Biological Bulletin*, **81**: 292.

cockroach by Alexandrowicz (1926) while comparable cells seem to have migrated out to part of the stomatogastric system in Aeschna (Zawarzin, 1911). Walling (1908) described cells which may be nervous in the heart of the grasshopper, Melanoplus. Few insects have been investigated and it may well be that some have myogenic hearts (Maloeuf, 1935). In decapod Crustacea, insects, and Limulus extrinsic regulating nerves have been described. In each of these groups there is little doubt from the location, the shape and size of the ganglion cells, and from operative experiments (particularly on Ligia and Limulus) that these nerve cells constitute the normal pacemakers. In each of these groups acetylcholine stimulates the pacemaker to faster discharge. These pacemaker cells are essentially autonomic ganglia; in the vertebrates postganglionic sympathetic neurones are excited by acetylcholine. In both arthropods and vertebrates, therefore, autonomic ganglia are excited by acetylcholine and in the higher arthropods where these ganglia are the cardiac pacemakers the heart is accelerated.

Recent evidence (Prosser and Zimmerman, 1942) places the hearts of some annelids in the group of acetylcholine-accelerated, neurogenic hearts.

Whether or not there are nerve cells in the hearts of molluscs has long been disputed. Dogiel (1877) described "apolar" nerve cells in several species; these could not function as normal neurones. Darwin (1876), Foster and Dew-Smith (1877) denied that these are nerve cells and Ransom (1883) showed these cells of Dogiel to be either plasma cells or connective tissue cells. These authors, as well as Motley (1933) and Esser (1934) were unable to find ganglion cells in the hearts of the molluscs they investigated. Suzuki (1934) described in the oyster heart cells which had processes like nerve cells, but he states that they were best seen in haematoxylin preparations which were hardly specific for nerve tissue. Alexandrowicz (1913) briefly stated that there are nerve cells in the heart of Octopus but did not describe them. Carlson (1905) summarized a great deal of evidence indicating that in all groups of molluscs there are cardioregulator nerves. In some forms these nerves are interrupted by ganglia which may be located directly on the heart (particularly in cephalopods). These ganglia contain secondary regulating neurones and can be removed without stopping the heart (Ransom, 1883). In most molluscs the beat can be seen to originate periodically at different points over the heart. It seems likely, therefore, that many cells which have been described as nerve cells in molluscan hearts are either blood cells or connective tissue cells, or are secondary neurones, and that all molluscan hearts are myogenic.

Table II shows that the hearts of all molluscan classes investigated are inhibited by acetylcholine.

The beat of the vertebrate heart arises in the sinus or in the sinauricular node which is muscular in nature (His, 1894, et al.). The myogenicity of the beat was proven by Gaskell (1900) who has been amply supported by later investigations. Nerve cells have been recognized in the hearts of all classes of vertebrates; they are distributed in the sinauricular region, over the auricle and near the auriculo-ventricular fissure. (See Woollard, 1926, for recent data and references.) Gaskell and Langley proved these to be secondary vagal neurones.

In both the molluscs and vertebrates, therefore, the heart is myogenic but innervated, and is inhibited by acetylcholine. Baylor (1942) has found the heart of Daphnia to be inhibited by acetylcholine. Ingle (personal communication), after using a variety of nerve-staining methods, has failed to find nerve cells in the heart of Daphnia. This arthropod may, therefore, have mutated to myogenicity.

Waterman (1942) studied the effect of acetylcholine on the heart of intact specimens of the ascidian, Perophora. He found the duration and number of beats of the abvisceral phase to be increased, and relative dominance of this phase over the advisceral phase to be strengthened by acetylcholine. Waterman has given me the opportunity to calculate heart rates from his original data. In each of six experiments with acetylcholine and in two with mecholyl a smooth marked acceleration in rate occurs. The frequency in beats per minute for successive abvisceral phases each lasting $1\frac{1}{2}$ to 2 minutes in one typical experiment follows: in sea water 56.7, 55.2, 55.6; then in acetylcholine (10^{-5}) 50.8, 64.3, 64.1, 62.1, 66.6, 65.3, 66.8. Acceleration occurs in both pacemakers but the threshold for the abvisceral one is lower than that for the advisceral beat. Bacq (1934a, 1935) stated that in physiological concentrations of acetylcholine there is no effect on the heart of Ciona but that strong concentrations stop the heart in systole and on recovery the beat is faster. Schultze (1901) and Ransom (1883) failed to find ganglion cells in hearts of Salpa; Alexandrowicz (1913) claimed to find them in Ciona but gave no figures. Hunter (1902) figured bipolar ganglion cells at the ends of the Molgula heart. According to the relation between acetylcholine acceleration and neurogenicity presented in this paper, Waterman's evidence supports Hunter that at least some ascidian hearts are neurogenic. One must conclude either that the ascidians show aberrant mutation from the chordate line or else arose long before the main chordate characters were established. In support of the latter view is the fact that ascidian

muscles contain the invertebrate arginine phosphate rather than the vertebrate phosphocreatine (Baldwin, 1937).

The embryonic Fundulus heart is insensitive to acetylcholine until the vagal secondary neurones have migrated to the heart (Armstrong, 1935). The chick heart is relatively insensitive to muscarine until innervation at about 100 hours (Pickering, 1893). We have shown above that the embryonic Limulus heart is unaffected by acetylcholine. It seems likely, therefore, that a non-innervated heart, whether it is to be myogenic or neurogenic, is insensitive to acetylcholine. Apparently either innervation sensitizes the heart or else acetylcholine acts at the nerve terminations. If this reasoning is correct, the hearts of Artemia and Eubranchipus are non-innervated. This view is supported by the fact that locomotor rhythms in Artemia could be altered by pressure and by eserine without appreciably affecting the heart rate.

Wherever it has been tested, potassium acts in the same direction as acetylcholine, inhibiting myogenic and accelerating neurogenic hearts. Calcium slows neurogenic hearts. Its action on myogenic hearts is complicated by its effects on the contractile system. Atropine antagonizes acetylcholine, presumably by competing for the receptor substances, in vertebrates and decapod Crustacea, but in most molluscs (oyster, Jullien, 1936; Loligo, Bacq, 1934b; Aplysia, Heymans, 1924; Venus, Prosser 1940b, et al.) there are toxic effects and no antagonism is found. This may mean that the receptor substances in the heart cells are different in vertebrates and molluscs. Muscarine also yields conflicting results; it accelerates in Cancer (Davenport, 1942) and inhibits in Limulus (Nukada, 1917); it initially accelerates in Mya (Yung, 1881) and inhibits in cephalopods (Ransom, 1883). Nicotine usually stimulates neurogenic hearts; its effect on myogenic hearts varies greatly with concentration. It thus becomes difficult, if not impossible, to classify acetylcholine effects on hearts as muscarine-like or nicotine-like. Adrenalin accelerates all those hearts which it affects. The hearts of Artemia (this paper), Ciona (Bacq, 1934a) and Nereis (Federighi, 1928) are unaffected by it. Many molluscan hearts are relatively insensitive to adrenalin (Boyer, 1926; Motley, 1934, et al.). Its effect upon non-innervated embryonic hearts seems not to have been tested.

The evidence summarized in Table II shows that hearts fall into three classes with respect to the action of acetylcholine, and suggests that those hearts which are accelerated are neurogenic, those which are inhibited are innervated myogenic, and those which are unaffected are non-innervated.

TABLE II

Animal	*Acetylcholine action*	*Presence of ganglionic pacemaker*
Annelida		
Lumbricus	+ (Prosser and Zimmerman, 1942)	Yes (Stubel, 1909)
Arenicola	+ (Prosser and Zimmerman, 1942)	Yes (Carlson, 1908)
Arthropoda		
Crustacea		
Decapoda		
Carcinus	+ (Welsh, 1939*a*)	Yes (Alexandrowicz, 1932)
Maia	+ (Welsh, 1939*a*)	Yes (Alexandrowicz, 1932)
Panulirus	+ (Welsh, 1939*b*)	Yes (Alexandrowicz, 1932) Yes (Welsh, 1939*b*)
Cancer	+ (Davenport, 1941)	Yes (Alexandrowicz, 1932)
Astacus and Cambarus	+ (Davenport et al., 1940)	Yes (Berger, 1877)
	+ (MacLean and Beznak, 1933)	Yes (Dogiel, 1894)
	+ (Prosser, this paper)	Yes (Nevomywaka, 1928)
Potamobius		Yes (Alexandrowicz, 1932)
Homarus	+ (Welsh, 1940, 1942)	Yes (Alexandrowicz, 1932)
Libinia	+ (Prosser [3])	Yes (Smith by Prosser, 1940)
Squilla		Yes (Alexandrowicz, 1934)
Palaemon		Yes (Nusbaum, 1899)
Amphipoda		
Bactrurus	+ (Prosser, this paper)	
Talorchestia	+ (Prosser, this paper)	
Isopoda		
Ligia		Yes (Alexandrowicz, 1931)
Asellus	+ (Prosser, this paper)	
Copepoda		
Diaptomus	+ (Prosser, this paper)	
Branchiopoda		
Daphnia	− (Baylor, 1942)	No (Ingle, personal communication)
Artemia	0 (Prosser, this paper)	
Eubranchipus	0 (Prosser, this paper)	
Xiphosura		
Limulus	+ (Garrey, 1941, 1942)	Yes (Patten and Redenbaugh, 1899) Yes (Carlson, 1904)
Limulus embryo	0 (Prosser, this paper)	No (Carlson and Meek, 1908)
Insecta		
Periplaneta [4]		Yes (Alexandrowicz, 1926)
Melanoplus	+ (Hamilton, 1939)	Yes? (Walling, 1908)
Aeschna		Probably migrated outside heart (Zawarzin, 1911)

[3] Experiments unreported but used regularly for class demonstration.

[4] Mr. Herbert B. Saslow has recently found in my laboratory that the heart of the cockroach, *Blatta orientalis*, is reversibly accelerated by low concentrations of acetylcholine. He also finds that the heart of the honeybee is similarly affected.

TABLE II—*Continued*

Animal	*Acetylcholine action*	*Presence of ganglionic pacemaker*
Mollusca		
Gastropoda		
Helix	− (Jullien, 1936a) − (Jullien et al., 1939)	No (Darwin, 1876) No (Foster and Dew-Smith, 1877) No (Ranson, 1883) Apolar cells [5] (Pompilian, 1900) No (Alexandrowicz, 1913)
Murex	− (Jullien, 1936a)	
Ariolimax	− (Davenport et al., 1940)	
Aplysia	− (Heymans, 1924) − (Jullien, 1937)	Apolar cells (Dogiel, 1877)
Pterotrachea		No (Ransom, 1883)
Pelecypoda		
Pecten		Apolar cells (Dogiel, 1877)
Mytilus	− (Jullien et al., 1938)	
Anadonta and other mussels	− (Prosser [5])	Apolar cells (Dogiel, 1877) No (Motley, 1933) No (Esser, 1934)
Ostrea	− (Jullien, 1936b)	Yes? (Suzuki, 1934)
Venus	− (Prosser, 1940)	No (Smith by Prosser, 1940)
Cephalopoda		
Octopus	muscarine − (Ransom, 1883)	Yes? (Alexandrowicz, 1913) No (Ransom, 1883, ganglion of secondary neurones)
Sepia	− (Kruta, 1936)	
Loligo	− (Bacq, 1934*b*)	Ganglion of secondary neurones (Carlson, 1905)
Chordata		
Urochorda		
Salpa		No (Ransom, 1883) No (Schultze, 1901)
Ciona	0+ (Bacq, 1934*a*, 1935)	No (Ransom, 1883) Yes? (Alexandrowicz, 1913)
Molgula		Yes (Hunter, 1902)
Perophora	+ (Waterman, 1942)	
Vertebrata		
Probably all classes	− (Dale, 1914) − (Hunt, 1915)	Secondary vagal neurones (Woollard, 1926, Gaskell, 1900 for references)
Fundulus embryo	0 (Armstrong, 1935)	pre-innervation stage
Chick embryo	muscarine 0 (Pickering, 1893)	pre-innervation stage

The effect of acetylcholine on the hearts of animals named and the presence or absence of ganglionic pacemaker. 0 indicates no effect, − inhibition, and + acceleration.

[5] Experiments unreported but used regularly for class demonstration.

Summary

The effects of drugs upon the hearts of a number of arthropods have been investigated by immersion of the specimens in test solutions. Acetylcholine, potassium and other substances affect the heart of a crayfish when they enter through the body surface (gills) although the threshold is much higher than when the drugs are applied directly to the heart.

Two amphipods, the marine *Talorchestia longicornis* and the blind freshwater *Bactrurus mucronatus* show cardiac acceleration by acetylcholine. The heart of the blind freshwater isopod *Asellus tridentatus* is accelerated by acetylcholine as are the hearts of the copepods *Diaptomus oregonensis* and *Diaptomus sanguinensis*.

The heart of *Artemia salina* is unaffected by acetylcholine even in very high concentrations, with or without eserine. Eserine alone has a toxic depressing action on Artemia. The Eubranchipus heart is also unaffected by acetylcholine. Dye penetration and injection experiments show that the lack of acetylcholine action on Artemia is not due to lack of penetration.

Pure adrenalin is without effect on the Artemia hearts, but adrenalin solution (Parke, Davis) markedly inhibits due to the reducing agent sodium bisulfite.

Limulus embryos during the myogenic period of heart contraction (21 to 33 days of development) show no effect of acetylcholine. Acceleration appears along with innervation.

A comparison of the effects of acetylcholine with the presence of extrinsic innervation and of ganglionic pacemakers shows the following: The hearts of higher arthropods and of some annelids and tunicates which are accelerated are neurogenic. The hearts of adult vertebrates, of molluscs and probably Daphnia which are inhibited by acetylcholine are myogenic but innervated. Embryonic hearts of vertebrates, Limulus, and the hearts of Artemia and Eubranchipus are unaffected by acetylcholine and are probably non-innervated.

LITERATURE CITED

ALEXANDROWICZ, J. S., 1913. Zur Kenntnis des sympathischen Nervensystems einiger Wirbellosen. *Zeit. f. allg. Physiol.*, **14**: 358–376.

ALEXANDROWICZ, J. S., 1926. The innervation of the heart of the cockroach (Periplaneta orientalis). *Jour. Comp. Neur.*, **41**: 291–309.

ALEXANDROWICZ, J. S., 1931. Quelques expériences sur le fonctionnement du système nerveux du coeur des Crustacés Isopodes. *Compt. Rend. Soc. Biol.*, **108**: 1270–1272.

ALEXANDROWICZ, J. S., 1932. Innervation of the hearts of Crustacea. I. Decapoda. *Quart. Jour. Micr. Sci.*, **75**: 181–249.

ALEXANDROWICZ, J. S., 1934. Innervation of the hearts of Crustacea. II. Stomatopoda. *Quart. Jour. Micr. Sci.*, **76**: 511–548.

ARMSTRONG, P. B., 1935. The role of the nerves in the action of acetylcholine on the embryonic heart. *Jour. Physiol.*, **84**: 20–32.

BACQ, Z. M., 1934a. Observations physiologiques sur le coeur, les muscles et le système nerveux d'une Ascidie (Ciona intestinalis). *Acad. Roy. Belg. Bull. Cl. Sci.*, **20**: 1042–1061.

BACQ, Z. M., 1934b. Réactions du ventricule médian, des chromatophores et de divers organes isolés d'un mollusque céphalopode (Loligo pealii) a l'adrénaline, l'acetylcholine, l'ergotamine, l'atropine et aux ions K, Ca et Mg. *Arch. Int. d. Physiol.*, **38**: 138–159.

BACQ, Z. M., 1935. Observations physiologiques sur le coeur, les muscles et le système nerveux d'une Ascidie. *Arch. Int. d. Physiol.*, **40**: 357–373.

BALDWIN, E., 1937. An introduction to comparative biochemistry. Camb. Univ. Press. 112 pp.

BAYLOR, E. R., 1942. Cardiac pharmacology of the cladoceran, Daphnia. *Biol. Bull.*, **83**: 165–172.

BERGER, EMIL, 1877. Über das Vorkommen von Ganglienzellen im Herzen vom Flusskrebs. *Sitzungsber. der K. Akad. der Wiss. Wien, Math.-Nat. Klasse*, **74**: 422–424.

BOYER, P., 1926. Action de l'adrénaline et de la spartéine sur le coeur de l'Escargot. *Compt. Rend. Soc. Biol.*, **95**: 1244–1247.

CARLSON, A. J., 1904. Nervous origin of heart-beat in Limulus and the nervous nature of coordination or conduction in the heart. *Am. Jour. Physiol.*, **12**: 67–74.

CARLSON, A. J., 1905. The innervation of the invertebrate heart. *Biol. Bull.*, **8**: 123–168.

CARLSON, A. J., 1908. Comparative physiology of the invertebrate heart. X. A note on the physiology of the pulsating blood vessels in the worms. *Am. Jour. Physiol.*, **22**: 353–356.

CARLSON, A. J., AND W. J. MEEK, 1908. On the mechanism of the embryonic heart rhythm in Limulus. *Am. Jour. Physiol.*, **21**: 1–10.

CROZIER, W. J., AND T. J. B. STIER, 1927. Temperature and frequency of cardiac contractions in embryos of Limulus. *Jour. Gen. Physiol.*, **10**: 501–518.

DALE, H. H., 1914. The action of certain esters and ethers of choline, and their relation to muscarine. *Jour. Pharm. and Exp. Therap.*, **6**: 147–190.

DARWIN, FRANCIS, 1876. On the structure of the snail's heart. *Jour. Anat. and Physiol.*, **10**: 506–510.

DAVENPORT, D., 1941. Effects of acetylcholine, atropine, and nicotine on the isolated heart of the commercial crab, Cancer magister Dana. *Physiol. Zool.*, **14**: 178–185.

DAVENPORT, D., 1942. Further studies in the pharmacology of the heart of Cancer magister Dana. *Biol. Bull.*, **82**: 255–260.

DAVENPORT, D., J. W. LOOMIS, AND C. F. OPLER, 1940. Notes on the pharmacology of the hearts of Ariolimax columbianus and Astacus trowbridge. *Biol. Bull.*, **79**: 498–507.

DOGIEL, J., 1877. Die Muskeln und Nerven des Herzens bei einigen mollusken. *Arch. f. mik. Anat.*, **14**: 59–65.

DOGIEL, J., 1894. Beitrag zur vergleichenden Anatomie und Physiologie des Herzens. *Arch. f. mik. Anat.*, **43**: 223–239.

ESSER, W., 1934. Recherches sur la structure des fibres musculaires cardiaques et sur l'innervation intrinsèque du coeur de l'Anadonte. *Arch. de Biol.*, **45**: 377–390.

FEDERIGHI, H., 1928. Blood vessels of annelids. *Jour. Exp. Zool.*, **50**: 257–294.

FOSTER, M. AND A. G. DEW-SMITH, 1877. Die Muskeln und Nerven des Herzens bei einigen Mollusken. *Arch. f. mik. Anat.*, **14**: 317–321.

GARREY, W. E., 1941. Action of acetylcholine on the heart of Limulus polyphemus. *Am. Jour. Physiol.*, **133**: P 288.

GARREY, W. E., 1942. An analysis of the action of acetylcholine on the cardiac ganglion of Limulus polyphemus. *Am. Jour. Physiol.*, **136**: 182–193.

GASKELL, W. H., 1900. The contraction of cardiac muscle. Pp. 169–227 in Schäfer's Textbook of Physiology, vol. 2.

HAMILTON, H. L., 1939. The action of acetylcholine, atropine and nicotine on the heart of the grasshopper (Melanoplus differentialis). *Jour. Cell. Comp. Physiol.*, **13**: 91–104.

HEYMANS, C., 1924. Influence des ions et de quelques substances pharmacodynamiques sur le coeur d'Aplysia limacina. *Arch. Internat. d. Pharm. e.d. therapie*, **28**: 337–347.

HIS, W., 1894. Herzmuskel und Herzganglien. *Wiener Medizin. Blätter*, **17**: 653–655.

HUNT, R., 1915. A physiological test for choline and some of its applications. *Jour. Pharm. and Exp. Therap.*, **7**: 301–337.

HUNTER, G. W., 1902. The structure of the heart of Molgula manhattensis (Verrill). *Anat. Anz.*, **21**: 241–246.

JULLIEN, A., 1936a. Des réactions comparées des coeurs de Vertébrés et d'Invertébrés vis-à-vis des électrolytes et des drogues. Paris. Baillière. 210 pp.

JULLIEN, A., 1936b. De l'action de certains poisons sur le coeur de l'huitre et des mollusques en général. *Jour. de Physiol. e.d. Path. Gen.*, **34**: 774–789.

JULLIEN, A., 1937. Action diastolisante de l'acétylcholine appliquée à l'étude de l'architecture et de l'automatisme cardiaques chez Aplysia fasciata. *Compt. Rend. Soc. Biol.*, **124**: 650–652.

JULLIEN, A., AND D. VINCENT, 1938. Sur l'action de l'acétylcholine sur le coeur des mollusques. L'antagonisme curare-acétylcholine. *Compt. Rend. Acad. Sci. Paris*, **206**: 209–211.

JULLIEN, A., D. VINCENT, M. VUILLET, AND M. BOUCHET, 1939. Contribution a l'étude de l'automatisme cardiaque chez les mollusques. Action de mimétiques et de drogues sur le coeur d'"Helix pomatia." *Jour. de Physiol. e.d. Path. Gén.*, **37**: 562–572.

KROGH, A., 1939. Osmotic regulation in aquatic animals. Cambridge. 242 pp.

KRUTA, V., 1935. Sur l'action d'acétylcholine et de l'atropine sur le coeur de Sepia officinalis. *Compt. Rend. Soc. Biol.*, **119**: 608–610.

MACLEAN, M. N., AND A. B. L. BEZNAK, 1933. The effect of sympatheticomimetic and parasympatheticomimetic drugs upon the hearts of decapod Crustacea. *Arb. Ungar. Biol. Forschungs.*, **6**: 258–263. (by abstract)

MALOEUF, N. S. R., 1935. Myogenic automatism of contraction of the heart of insects. *Ann. Ent. Soc. Amer.*, **28**: 332–337.

MALUF, N. S. R., 1940. The uptake of inorganic electrolytes by the crayfish. *Jour. Gen. Physiol.*, **24**: 151–167.

MOTLEY, H. L., 1933. Histology of the fresh-water mussel heart with reference to its physiological reactions. *Jour. Morph.*, **54**: 415–427.

MOTLEY, H. L., 1934. Physiological studies concerning the regulation of heartbeat in fresh-water mussels. *Physiol. Zool.*, **7**: 62–84.

NEVOMYWAKA, G. A., 1928. Zur Frage über die Innervation des Herzens beim Flusskrebs (Potamobius astacus L.). *Zool. Anz.*, **79**: 209–222.

NUKADA, S., 1917. Über die Automatie und die Koordination des Herzens (Biologische Untersuchungen über das Herz des Limulus longispina). *Mitt. a. d. Med. Fak. d. Kaiser. Univ. z. Tokyo*, **19**: 1–164.

NUSBAUM, J., 1899. Beiträge zur Kenntnis der Innervation des Gefäss-systems nebst einigen Bemerkungen über das subepidermale Nervenzellengeflecht bei den Crustaceen. *Biol. Centr.*, **19**: 700–711.

PATTEN, W., AND W. A. REDENBAUGH, 1899. Studies on Limulus. II. The nervous system of Limulus polyphemus, with observations upon the general anatomy. *Jour. Morph.*, **16**: 91–200.

PICKERING, J. W., 1893. Observations on the physiology of the embryonic heart. *Jour. Physiol.*, **14**: 383–466.

POMPILIAN, M., 1900. Cellules nerveuses du coeur de l'Escargot. *Compt. Rend. Soc. Biol.*, **52**: 185–187.

PROSSER, C. L., 1940a. Action potentials in the nervous system of the crayfish. Effects of drugs and salts upon synaptic transmission. *Jour. Cell. Comp. Physiol.*, **16**: 25–38.

PROSSER, C. L., 1940b. Acetylcholine and nervous inhibition in the heart of Venus mercenaria. *Biol. Bull.*, **78**: 92–102.

PROSSER, C. L., AND G. ZIMMERMAN, 1942. Effects of drugs on the hearts of Arenicola and Lumbricus. In press.

RANSOM, W. B., 1883. On the cardiac rhythm of invertebrates. *Jour. Physiol.*, **5**: 261–341.

SCHULTZE, L. S., 1901. Untersuchungen über den Herzschlag der Salpen. *Jen. Zeit. f. Naturw.*, **35**, **N.F. 28**: 221–328.

SUZUKI, S., 1934. On the distribution of ganglion cells in the heart of the pearl oyster. *Sci. Rep. Tohoku Imp. Univ. Ser. 4 (Biol.)* **8**: 335–344.

STÜBEL, H., 1909. Studien zur vergleichenden Physiologie der peristaltischen Bewegungen. IV. Die Peristaltik der Blutgefässe des Regenwurmes. *Pflüg. Arch. f. d. ges. Phys.*, **129**: 1–34.

WALLING, L. V., 1908. The anatomy of the Acrididean heart and its histological structure. *Kansas Univ. Sci. Bull.*, **4**: 359–367.

WATERMAN, A. J., 1942. The action of drugs on the compound ascidian Perophora viridis, as indicated by the activity of the intact heart. *Physiol. Zool.*, **15**: 61–74.

WELSH, J. H., 1939a. Chemical mediation in crustaceans. I. The occurrence of acetylcholine in nervous tissues and its action on the decapod heart. *Jour. Exp. Biol.*, **16**: 198–219.

WELSH, J. H., 1939b. Chemical mediation in crustaceans. II. The action of acetylcholine and adrenalin on the isolated heart of Panulirus argus. *Physiol. Zool.*, **12**: 231–237.

WELSH, J. H., 1940. The action of acetylcholine on the lobster heart. *Anat. Rec.*, **78** Supp.: 68.

WELSH, J. H., 1942. Chemical mediation in crustaceans. IV. The action of acetylcholine on isolated hearts of Homarus and Carcinides. *Jour. Cell. Comp. Physiol.*, **19**: 271–279.

WOOLLARD, H. H., 1926. The innervation of the heart. *Jour. Anat.*, **60**: 345–373.

YUNG, E., 1881. De l'innervation du coeur et de l'action des poisons chez les Mollusques lamellibranches. *Arch. Exp. d. Zool.*, **9**: 429–444.

ZAWARZIN, A., 1911. Histologische Studien über Insekten. I. Das Herz der Aeschnalarven. *Zeit. f. wiss. Zool.*, **97**: 481–510.

CARDIAC PHARMACOLOGY OF THE CLADOCERAN, DAPHNIA

E. R. BAYLOR

(Department of Zoology, University of Illinois, Urbana)

Acetylcholine has been demonstrated to speed the heart of several of the decapod crustacea (Welsh, 1939 a and b; Davenport, Loomis and Opler, 1940 and Davenport, 1941), the grasshopper (Hamilton, 1939), and Limulus (Garrey, 1941). Is this reaction, which may be indicative of a neurogenic heart, a general characteristic of the arthropods? The pharmacology of the heart of the cladoceran, Daphnia, was examined to extend the observations to one of the lower groups within the phylum.

Obreshkove, 1941, has demonstrated the action of acetylcholine, eserine, and atropine on intestinal movement in Daphnia. Acetylcholine stimulates intestinal movement, its action is inhibited by atropine and potentiated by eserine.

Materials and Methods

Young animals, largely of the second instar of the species *Daphnia magna*, were used in this work to insure uniform age. At this stage, the animals are quite transparent and generally amenable to the requirements of the experiments. Older animals reacted in the same manner as younger ones but they were not used so extensively because they were more opaque.

At room temperatures the heart beat of Daphnia is too rapid to count with the naked eye. Therefore a stroboscope was devised to measure the cardiac rate at these higher temperatures. In other experiments the specimen was cooled to 10° C. and the heart was counted by eye.

Essentially the stroboscope consisted of a shaft with a light interrupter disc and a tachometer, all driven by a variable speed motor. The interrupter disc cut the light beam from a Spencer microscope lamp as it impinged on the mirror of a microscope used for viewing the preparation. The tachometer was a motor generator which was calibrated so that revolutions per minute could be obtained directly from reading the current generated.

Visual counting was made possible by lowering the temperature to 10° ± 0.2. At this temperature it was possible to obtain the rate directly and accurately with a stop watch.

The standard procedure was to place the animal upon a network of cotton fibers in a depression slide of about 0.5 cc. volume. The experimental fluids could then be applied and removed by means of two pipettes, one of which held the test solution and the other of which was connected to a vacuum source for efficient removal of the solution. Counts of the heart rate were made after equilibration in dechlorinated tap water. The experimental solutions were then added and the heart rate was followed. Finally, the dechlorinated tap water was replaced on the animal to allow recovery. In order to assure the concentration of any solution employed, the preparation was washed several times with the solution and the entire volume was changed at least four times.

The adrenalin used in these experiments was prepared by Parke, Davis and Company; atropine sulfate and eserine sulfate (physostigmine) were prepared by Hoffman-La Roche; acetylcholine bromide by Eastman Kodak Company.

The solutions were made up to the indicated strengths in aerated non-chlorinated tap water.

Results

The normal heart rate varies from four beats per second to ten beats per second at room temperature. Feeding causes great variations. Animals of a culture might show an average beat of four beats per second before being fed, and a day later after receiving yeast show a beat of ten per second. The large culture jars were aerated continuously. It was found that while in the depression slide additional aeration was not necessary and that the beat would maintain itself for four to five hours without it.

Acetylcholine

The results obtained from forty experiments with acetylcholine agree in showing that acetylcholine inhibits the heart rate in Daphnia. Figure 1 *A*, *B*, *C*, *D*, shows the results on single animals using a series of different concentrations. There is a direct relation between the heart rate and the concentration of the drug. The threshold appears to be 10^{-9} at which concentration one of three experiments gave a 3 per cent inhibition, the others none. A concentration of 10^{-8} in three experiments gave an average inhibition of 8 per cent; 10^{-7} in five experiments gave an average inhibition of about 18 per cent; 10^{-6} in

six experiments gave an inhibition averaging 21 per cent; and 10^{-5} in four experiments gave an average inhibition of 27 per cent.

As can be seen by the graphs, the effect of acetylcholine is not completely reversible in every case. In some cases after dechlorinated

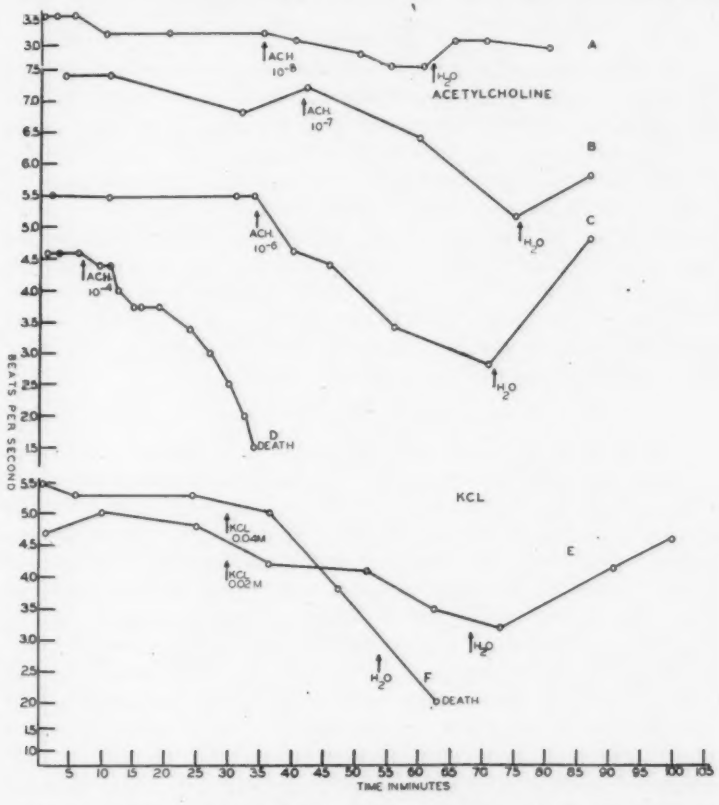

FIG. 1. Effects of acetylcholine (*A B, C, D*) and potassium (*E, F*) on the heart rate in *Daphnia magna*. Specimens placed in solutions as indicated by arrows. Ach, acetylcholine, H_2O dechlorinated tapwater.

water was added following retardation, the rate rose to only one-half the original rate. This indicates a toxic effect of acetylcholine on the heart of Daphnia, particularly at higher concentrations.

Acetylcholine in higher concentrations (10^{-6} to 10^{-4}) causes a series of events starting with a slowing of the heart rate followed by a

weakening of the strength of the beat. There is always observed a great reduction of amplitude in these concentrations. Next a sort of doubling occurs which is probably the result of the wave of contraction not being conducted completely over the heart. In a few cases, such as that seen in Figure 1 *D*, the heart stopped in diastole.

Atropine

Atropine was applied in fifteen experiments in concentrations from 10^{-8} to 10^{-2}. In these concentrations it causes a negative chronotropic effect which is probably a toxic one since the addition of water failed to bring about recovery from solutions higher than 10^{-8}. Concentrations of 10^{-7} to 10^{-5} inhibited by from 6 to 8 per cent and 10^{-2} inhibited by 15 per cent. After replacement of the atropine by water the heart beat did not return to its normal rate but remained low for many hours, after which the animals died. Experiments on the antagonism of acetylcholine by atropine, such as was observed by Obreshkove, were thus impossible to perform. These results do not agree with those obtained by Pickering, 1894, on Daphnia. He found that atropine accelerated the heart rate.

Eserine

Eserine on the heart of Daphnia causes an inhibition which is not reversible upon addition of water. Two experiments were performed at each of the four concentrations. There was no effect at 10^{-9}. In any concentration higher than and including 10^{-8}, the heart beat was inhibited in varying amounts depending on the concentration of eserine. Addition of water did not bring about recovery and the heart rate remained low and eventually stopped. Because of this toxicity, experiments on eserine potentiation rendered results which could not be interpreted except in the light of the toxicity. Two experiments using subthreshold eserine followed by acetylcholine gave no indication of potentiation. Obreshkove (1941) working on intestinal movements found potentiation of acetylcholine by previous eserine treatment. It would therefore be expected that the heart would show similar reactions. Further experiments in which the time of exposure to eserine is much reduced before treatment with acetylcholine and the time factor of the appearance of inhibition is carefully noted, should be performed before any definite statement can be made.

Adrenalin

Application of commercial adrenalin (Parke, Davis solution) to the heart of Daphnia gave erratic results (Figures 2 *B* and *C*). This was shown by Prosser (1942) in the course of experiments on Artemia to be

due to the sodium bisulfite added to the commercial adrenalin solution to prevent auto-oxidation of the ardenalin. This was true for Daphnia as well. Comparable concentrations of $NaHSO_3$ (1% diluted to 10^{-7}) without adrenalin cause cardiac inhibition as high as 20 per cent. (Figures 2 *D* and *E*.) Solutions of pure crystalline adrenalin give

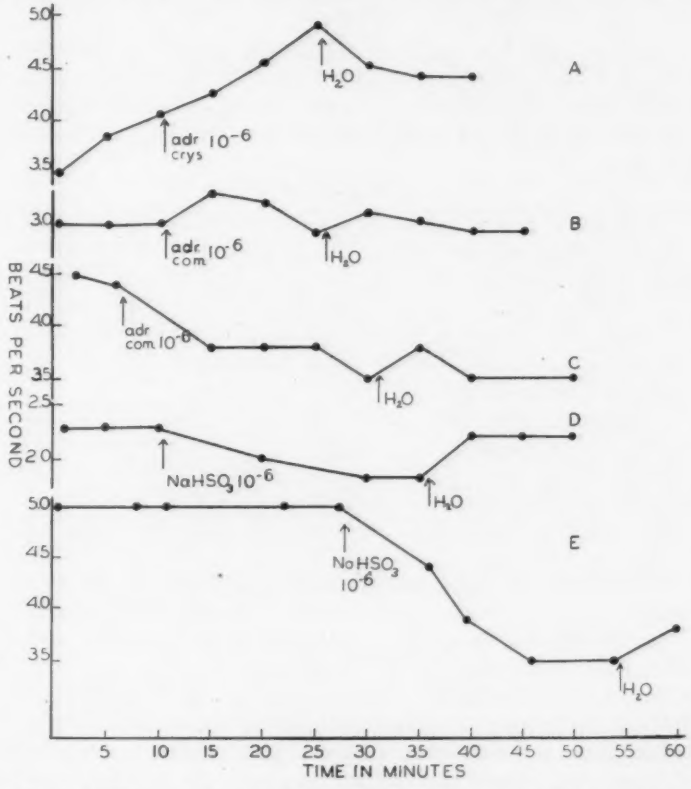

FIG. 2. Effects of: *A*, crystalline adrenalin (adr. crys.); *B*, *C*, commercial adrenalin solution (adr. com.) and *D*, *E*, sodium bisulfite on the heart rate of *Daphnia magna*.

notable accelerations which increase as the concentration increases (Figure 2 *A*).

Potassium

Potassium causes an inhibition in the rate of the beat. Eight experiments were performed. Concentration of 0.005 M was sub-

threshold; 0.01 M, in two experiments, caused an inhibition of 1.5 per cent; .02 M approximately 12 per cent (see Figures 1 *E* and *F*). The highest concentration used, 0.04 M, was toxic and did not reverse upon return to water (see Figure 1 *F*). This curve appears very similar to that of acetylcholine 10^{-4}.

Nicotine

Nicotine applied to the heart of Daphnia shows a stimulation in the weaker concentrations and an inhibition in the stronger concentrations which is not reversible. A concentration of 10^{-6} showed an acceleration of 6 per cent; 10^{-4} a 17 per cent fall; and 10^{-3} a 35 per cent fall.

Discussion

The heart of Daphnia, like the heart of the vertebrate, is inhibited by acetylcholine and potassium. In all arthropods previously considered except Daphnia there is an acceleration of the beat in response to acetylcholine and potassium. The hearts of all vertebrates appear, from histological evidence, to be myogenic and those of all decapod crustacea and Limulus appear from histological evidence to be neurogenic (Alexandrowicz, 1932; Carlson, 1904).

Obreshkove, 1941, has shown that stimulation of the gut of Daphnia by pricking causes increased peristalsis of the intestine and an inhibition of the heart. He also found similar increased peristalsis upon application of acetylcholine which, as shown by our experiments, causes a decreased heart rate. Acetylcholine in the vertebrate causes peristalsis and decreased heart rate. This, then, is a further indication of the similarity of the cholinergic systems of Daphnia and the vertebrates.

On the basis of the evidence presented in the literature, Prosser (1942) has postulated that all myogenic hearts are inhibited by acetylcholine and potassium and that all neurogenic hearts are accelerated by acetylcholine and potassium. Since the pharmacology of the Daphnia heart resembles that of the vertebrate in all respects thus far tested, and since there is no work to indicate the presence of nerve cells in this heart, we may conclude that the Daphnia heart is myogenic and predict that it will be found to have no pacemaker nerve cells. Extrinsic or regulating innervation is, however, probable.

It might be argued that in the experiments presented in this work acetylcholine affects the heart by way of the central nervous system of the Daphnia. However, the following facts oppose this argument. In the vertebrates injection of acetylcholine into the intact animal gives cardiac inhibition much like that found in isolated hearts. Also,

crayfish half immersed in solutions of acetylcholine so that any acetylcholine reaching the heart must enter through the gills and pass by way of the blood to the heart, show acceleration (Prosser, unpublished data). Furthermore, the concentrations employed in this work on Daphnia closely approximate those used by other workers on other animals where the drugs were applied directly to the hearts.

Summary

1. Acetylcholine was found to have a depressing effect on the heart rate and strength of beat of the heart of *Daphnia magna* with the threshold at 10^{-9} and a depression of 25 per cent at 10^{-5}. The effect is reversible.

2. Atropine has a depressing effect on the heart rate with the threshold at 10^{-7} and an inhibition of 15 per cent at 10^{-4}. The effect is not reversible.

3. Eserine with a threshold of 10^{-9} has a toxic effect on the heart of Daphnia causing irreversible inhibition at any higher concentration.

4. Adrenalin has an accelerating effect at 10^{-7} when prepared from the crystalline form. Commercial adrenalin solution may actually inhibit due to the reducing agent, $NaHSO_3$, which itself inhibits heart rate in concentrations as low as 10^{-6}.

5. Potassium chloride inhibits the heart rate with a threshold at 0.005 M, a 12 per cent inhibition at 0.02 M and death at 0.04 M.

6. The above facts would indicate that the heart of Daphnia more closely resembles the vertebrate heart than that of the higher crustacea in its pharmacology.

At this time I wish to express my gratitude in acknowledgment of the help and advice given me by Dr. C. L. Prosser.

LITERATURE CITED

Alexandrowicz, J. S., 1932. Innervation of the hearts of Crustacea. I. Decapoda. *Quart. Jour. Micr. Sci.*, **75**: 181–249.

Carlson, A. J., 1904. Nervous origin of heart beat in Limulus and the nervous nature of coordination or conduction in the heart. *Am. Jour. Physiol.*, **12**: 67–74.

Davenport, D., 1941. Effects of acetylcholine, atropine and nicotine on the isolated heart of the commercial crab, Cancer magister Dana. *Physiol. Zool.*, **14**: 178–185.

Davenport, D., J. W. Loomis and C. F. Opler, 1940. Notes on the pharmacology of the hearts of Ariolimax columbianus and Astacus trowbridgei. *Biol. Bull.*, **79**: 498–507.

Garrey, W. 1941. Action of acetylcholine on the heart of Limulus polyphemus. *Am. Jour. Physiol.*, **133**: P 288.

Hamilton, H. L., 1939. The action of acetylcholine, atropine and nicotine on the heart of the grasshopper (Melanoplus differentialis). *Jour. Cell. Comp. Physiol.*, **13**: 91–104.

OBRESHKOVE, V. 1941. The action of acetylcholine atropine and physostigmine on the intestine of Daphnia magna. *Biol. Bull.*, **81**: 105–113.

PICKERING, J. W., 1894. On the action of certain substances on the heart of Daphnia. *Jour. Physiol.* **17**: 357–363.

PROSSER, C. L., 1942. An analysis of the action of acetylcholine on hearts particularly in arthropods. *Biol. Bull.*, **83**: 145–164.

WELSH, J. H., 1939a. Chemical mediation in Crustacea. I. The occurrence of acetylcholine in nervous tissues and its action on the decapod heart. *Jour. Exp. Biol.*, **16**: 198–219.

WELSH, J. H. 1939b. Chemical mediation in Crustacea. II. The action of acetylcholine and adrenalin on the isolated heart of Panulirus argus. *Physiol. Zool.*, **12**: 231–237.

THE HYDROGEN ION CONCENTRATION OF THE CONTENT OF THE FOOD VACUOLES AND THE CYTOPLASM IN *AMOEBA* AND OTHER PHENOMENA CONCERNING THE FOOD VACUOLES[1]

S. O. MAST

(The Johns Hopkins University, Baltimore, and the Marine Biological Laboratory, Woods Hole)

INTRODUCTION

The content of the food vacuole has been studied by various investigators in a considerable number of different protozoa. All maintain that it becomes acid shortly after the food vacuole is formed and that it later becomes neutral or alkaline, and all appear to assume that acids and bases are secreted by the cytoplasm and poured into the vacuoles. For example, Greenwood and Saunders (1894) say: "The ingestion of solid matter, whatever its nature, stimulates the surrounding cell substance to secrete acid fluid"; Nirenstein (1905) says: "Wie man sieht bedarf die Frage nach dem Sinne der Säuresekretion noch durchaus der Aufklärung"; Howland (1928, p. 131) says: "This would indicate that the acid [in the food vacuole] is secreted by the surrounding protoplasm"; and Claff et al. (1941) say: "The initial acid production around the vacuole was stimulated by the closure of the vacuole." Similar statements have been made concerning the bases.

It is generally held that the base in the vacuoles functions in the digestion of food, but concerning the function of the acid there is marked diversity of opinion. Hemmeter (1896) contends that it serves to kill the ingested organisms. Claff et al. (1941) support this contention. They say: "The prey is killed simultaneously with a sudden release of an acid into the newly-formed food vacuoles." Howland (1928) also favors it, and she seems to hold that the acid aids digestion. She says (p. 133): "It is possible that the acid functions as a killing fluid within the vacuole. . . . All vacuoles in which disintegrating food lies in the center surrounded by vacuolar fluid, appear to be in the process of active digestion. . . . The pH value of such vacuoles, as uniformly indicated by brom cresol green, is 4.3 ± 0.1." Nirenstein (1905) does not agree with these contentions. He maintains that the

[1] I am indebted to Dr. R. A. Fennell and to Dr. W. J. Bowen for very efficient assistance in this work.

acid in the food vacuole is not strong enough to kill bacteria, but he asserts that it may serve to make other substances present toxic. He holds that digestion does not begin until after the substance in the vacuole has become alkaline, but he thinks the acid may function in preparation for digestion. He says: "So wäre daran zu denken, dass die Säure bei der Aktivierung des Profermentes eine Rolle spielt; ferner könnte der Säure die Bedeutung zukommen, native Eiweisstoffe zu koagulieren, woraus sich indirekt eine gewisse Wichtigkeit der Säure für Verdauung ergeben würde." Greenwood and Saunders (1894) also maintain that digestion does not begin until after the vacuole is alkaline. They say: "The outpouring of acid is unaccompanied by any digestive change in nutritive matter."

According to these views, then, the cytoplasm around the younger food vacuoles in protozoa secretes acids which are poured into them and possibly serve to kill and digest the ingested organisms, and the cytoplasm around the older vacuoles secretes bases which are poured into them and function in digestion. These views are very largely based on results obtained in observations on ciliates. If the contention that the cytoplasm adjoining the surface of the food vacuoles pours acids into some and bases into others holds for *Amoeba*, it is a most remarkable phenomenon, for in these organisms food vacuoles with content respectively acid and alkaline are often in close contact with each other, and apparently surrounded by the same kind of cytoplasm.

Materials and Methods

Amoeba proteus, *Amoeba dubia*, *Chilomonas paramecium* and *Colpidium striatum* were used almost exclusively in the following experiments. They were raised in Hahnert solution [2] containing rice or wheat. The observations were made as follows: Amoebae were taken from the cultures and passed through fresh portions of Hahnert solution until all the organisms on which they ordinarily feed were removed. They were left in this solution until nearly all the food vacuoles had disappeared, i.e., for 24 hours or longer. Some of them were then mounted between ridges of vaseline on slides and either living or dead food stained with various indicator dyes added. Others were stained with various dyes, then mounted, and unstained living or dead food added. Cover-glasses were put on the preparations, the amoebae studied under low and high magnifications, and the length of life of the ingested organisms and the changes in the color and the structures in them observed.

[2] Hahnert (1932) "KCl, 0.004 gm.; $CaCl_2$, 0.004 gm.; $CaH_4(PO_4)_2$, 0.002 gm.; $Ca_3(PO_4)_2$, 0.002 gm.; $MgHPO_4$, 0.002 gm.; H_2O, 1000 cc. (pH 6.5)."

In ascertaining the hydrogen ion concentration of the content of the food vacuoles, Clark buffers covering the range of the dye under consideration, and differing by 0.2 pH were put respectively into small test tubes and the same quantity of the dye added to each. The content of the food vacuole in the amoebae was then compared in color with the buffers in the test tubes. In some experiments other methods were also used; these are described below.

Neutral Red

Living organisms ingested

Chilomonads and colpidia were added to culture solutions containing neutral red, and amoebae prepared as described above. The amoebae ingested the chilomonads and colpidia freely, frequently two or more at a time. Some of the food vacuoles thus formed were observed continuously for an hour or longer and briefly from time to time for 24 hours.

The newly formed food vacuoles contained large quantities of fluid in which the ingested organisms swam violently about. The amount of this fluid gradually decreased until there was little left (Fig. 1); then the organisms in it usually became quiet suddenly, after which they soon became purplish pink like the color of buffer, pH 3.6 (containing neutral red), and then very gradually pinkish (pH 6); but the fluid in the vacuoles remained colorless. Later the fluid increased in amount (Fig. 1) and became distinctly yellow, but the organisms in it were still pinkish, indicating that the fluid was now alkaline (about pH 8) and the organisms in it acid or neutral (pH 6 to 7). The fluid and the organisms then gradually became darker until the former was brownish yellow and the latter reddish brown (pH 8.8 to 9).[3] The time required for these changes varied greatly. Some details are presented in the following records taken from my notes.

(1) Specimens of *Amoeba dubia* were put into Hahnert solution containing neutral red on a slide and left about an hour, then colpidia were added. A few minutes later (1:40 P.M.) one of the amoebae ingested a colpidium. This colpidium was continuously observed for several hours and its color compared with those of a series of buffer solutions containing neutral red.

The colpidium swam vigorously in the food vacuole for nine minutes, then suddenly stopped. No change had taken place in the color of the colpidium and the fluid around it was colorless.

[3] Results presented below demonstrate that the content of the food vacuole does not become more alkaline than approximately pH 7.3 and that neutral red is not an accurate indicator.

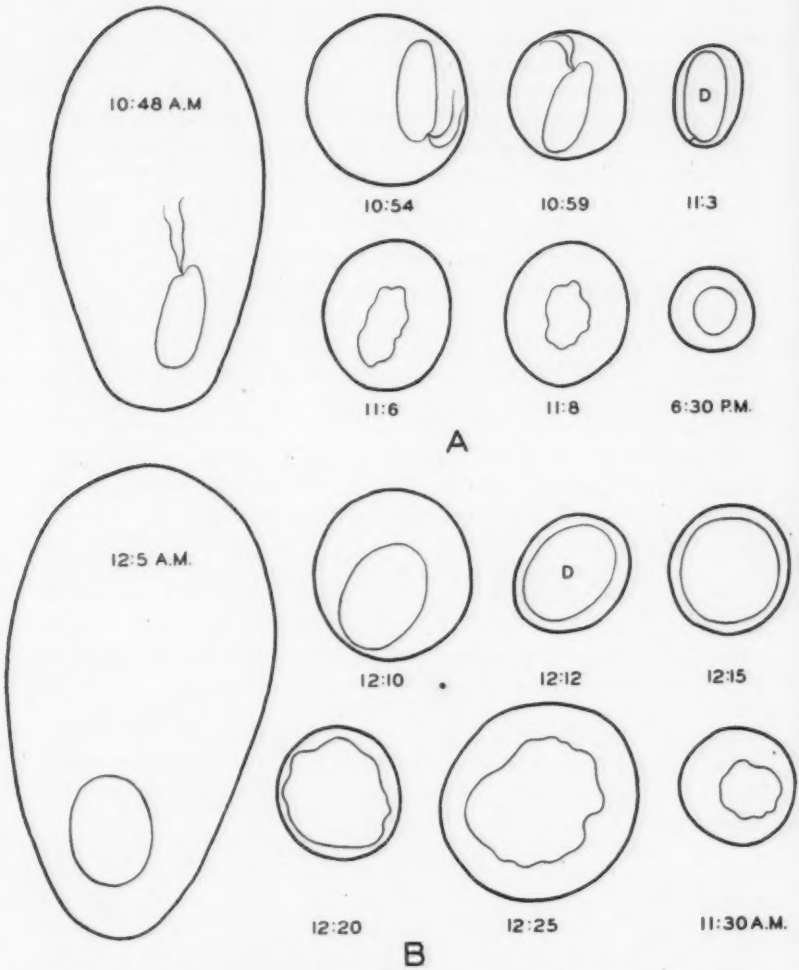

FIG. 1. Camera outlines showing changes in the size of food vacuoles containing organisms ingested by *Amoeba proteus*. *A*, food vacuole containing a chilomonad; *B*, food vacuoles containing a colpidium; numbers, time of day; *D*, approximate time the ingested organisms died.

Note that the vacuoles decreased greatly in size owing to the loss of fluid, then increased rapidly owing to absorption of fluid, and that the organisms died shortly after the fluid content became minimum.

(1:55 P.M.) Colpidium light purple, fluid colorless; (2:00) colpidium denser purple, about like buffer, pH 3.5, fluid colorless; (2:10 and 2:30) no change; (2:50) colpidium pinkish purple, pH 4.5 to 5, fluid colorless; (3:25) colpidium clearly more pinkish, pH 6, fluid colorless; (3:55) colpidium reddish pink, pH 6.6 to 7, fluid colorless; (4:50) colpidium more reddish, pH 7 to 7.5, fluid faintly yellowish, pH 8, clearly more alkaline than colpidium; (6:50) colpidium reddish yellow, pH 8, fluid brownish yellow, pH 8.8 to 9; (8:50) colpidium brownish yellow, fluid about the same.

(2) In another preparation containing chilomonads in place of colpidia the following was observed: (11:24 A.M.) Chilomonad ingested; (11:27) chilomonad dead, faintly purple, pH 3.5, fluid around chilomonad colorless; (11:32) several granules in the chilomonad, deep crimson, rest brilliant purple, pH 3.5, fluid colorless; (11:40) chilomonad definitely pinkish, about pH 7, fluid colorless; (12:30) chilomonad pink with slightly yellowish tint, about pH 7.6, fluid distinctly yellowish, pH 8; (1:30) chilomonad brownish yellow, pH 8+, fluid about the same.

Similar observations were made on many other food vacuoles containing colpidia and chilomonads respectively, some in *Amoeba proteus* and others in *Amoeba dubia*. The results obtained were essentially the same in all. The colpidia and chilomonads in all became distinctly purple almost immediately after they died, then gradually pinkish, then reddish, then brownish yellow. The fluid in the food vacuoles remained colorless until some time after the colpidia and chilomonads in it had become pink, then it became yellow, i.e., the organisms in the vacuoles remained acid for some time after the fluid around them had become alkaline. This shows that the base in the vacuoles originates, not in the ingested organisms but in the cytoplasm around the vacuoles.

The following observations show that the rapid increase in hydrogen ion concentration in organisms in food vacuoles is correlated with death. Colpidia and chilomonads were put into strong solutions of neutral red in Hahnert solution and observed closely. Very soon particles in the food vacuoles in the colpidia and a few granules in the cytoplasm of the chilomonads became red. Shortly after this some of the organisms died and then gradually more, until all were dead. Immediately after death the cytoplasm and the nucleus in all became purple, first very light then rapidly deeper, until the entire body was deep brilliant purple like buffer, pH 3 to 3.5, after which there was no change in color except that under some conditions it gradually faded out. This strongly indicates that the acid in the food vacuoles does not originate in the cytoplasm around them, but in the organisms in them.

The results presented above indicate that shortly after ingestion the organisms in the food vacuoles in *Amoeba* became decidedly acid, but they have no bearing on the question concerning the hydrogen ion concentration of the fluid in the vacuoles at this time. The following experiments concern this.

Neutral red crystals ingested

If a base is added to a neutral aqueous solution of neutral red, the solution becomes yellow and then needle-like brownish yellow crystals form. These crystals are readily soluble in most acid solutions. In oleic acid and probably also in some other fatty acids they round up and become very viscous and deep crimson in color.

The relation between the solubility of the crystals, the change in color and the hydrogen ion concentrations in different solutions was ascertained as follows: Crystals were washed in culture solution (pH 6.8), then a few in a very small amount of this solution were transferred to a number of relatively large quantities of solution differing in hydrogen ion concentration, and the effect noted.

Three different solutions were used: Clark buffers (acetate and phosphate, pH 4, 4.6, 5, 5.6, 6 and 6.6), Hahnert solution plus hydrochloric acid (pH 4, 4.5 and 5) and Hahnert solution plus lactic acid (pH 4, 4.5 and 5).

It was found that the crystals dissolve readily in Clark buffers at pH 5 and lower, and slightly at pH 5.6, and that in Hahnert solution plus lactic or hydrochloric acid they dissolve readily at pH 4 and lower, and slightly at pH 4.5, but somewhat more readily in the former than the latter. Details concerning two experiments follow:

(1) One cc. of each of the Clark buffers was put respectively into Columbia dishes on white paper, and a *very small* amount of Hahnert solution (pH 6.8) containing numerous crystals added. The crystals were then observed with the naked eye, and under low magnification. In buffers pH 4, 4.6 and 5, they became pink and dissolved immediately. In buffer pH 5.6 they became slightly pink at the surface in two minutes, and deep pink in six minutes. Some dissolved in 13 minutes, many in 20, nearly all in 40, and all in 60. In buffer pH 6, the crystals became faintly pink at the surface in 13 minutes, definitely pink in 20, and somewhat more strongly pink in 40. Observations were continued for four hours. Very few if any of the crystals dissolved.

(2) With Hahnert solution plus HCl in place of Clark buffers, pH 4, the crystals became definitely pink in one minute, and strongly pink in four. Nearly all dissolved in 14 minutes, and all in 24. In this solution, pH 4.5, they became slightly pink in four minutes and

definitely pink in 14. A few dissolved in 24 minutes but not all in four hours.

Specimens of *Amoeba dubia* were mounted in culture solution containing neutral red crystals, but no food organisms. The culture solution (Hahnert solution plus a little NaOH) was pH 6.8. The amoebae were active in this solution and appeared normal in all respects. Some of them soon ingested one or more crystals each (one ingested six). Several with crystals were closely followed from the time the crystals were ingested until four hours later. During this time the crystals moved freely with the plasmasol. Most of them appeared to be in close contact with the cytoplasm, not in vacuoles containing fluid. Some were ejected, others not. No change whatever was observed in any of these crystals, either in color or in size or in form.

This experiment was repeated twice with chilomonads added to the culture solution. Several of the amoebae ingested two to 15 chilomonads each, and one or more crystals, but none were found with chilomonads and crystals in the same food vacuoles. All were under close observation for several hours. No changes in any of the crystals were detected.

The experiment was also repeated twice with colpidia in the culture solution in place of chilomonads. In both tests the amoebae ingested colpidia promptly and in the formation of some food vacuoles crystals were taken in with the colpidia. Details concerning two such vacuoles in different amoebae follow:

(1) At 2:55 P.M. a colpidium was discovered violently swimming about in a food vacuole which contained a neutral red crystal. The movement of the colpidium greatly agitated the crystal, proving that it was actually in the fluid in the food vacuole. The food vacuole decreased rapidly in size. At 2:58 the colpidium came to rest and about one minute later it began to become purplish in color. At 3:03 it was very definitely purple (like buffer, pH 3.5) but the fluid in the vacuole was colorless and the crystal had not changed. At 3:20 the colpidium was purplish pink (pH 4.5 to 5) but the fluid was still colorless and no visible changes in the crystal had occurred. At 3:55 the vacuole had considerably increased in size. The colpidium was now pinkish red (pH 6.5) and the fluid around it light brownish yellow (clearly alkaline, about pH 8). The amoeba was lost soon after this, but in other specimens vacuoles were found in which the colpidia gradually changed color from pinkish red to brownish yellow, i.e., they became similar in color to the fluid around them.

(2) An amoeba was discovered forming a food cup over an area on the slide in which there were a colpidium, a large crystal, and an ag-

gregate of small pieces of crystals. As the mouth of the cup closed, the edge on all sides remained in close contact with the slide so that the crystals were taken in with the colpidium. The colpidium swam around in the cup, and the crystal and the aggregate of pieces were violently thrown about by the currents produced by the cilia. After a few moments the large crystal passed out of the vacuole into the cytoplasm and the aggregate became attached to the surface of the colpidium. A few moments later the colpidium became quiet and began to become purplish in color; the vacuole had decreased considerably in size, owing to loss of fluid. About two minutes later the colpidium was purplish pink (pH 3.5 to 4) but the fluid in the vacuole was still colorless and there was no visible change in the crystals. Thirty minutes later the vacuole had distinctly increased in size, the fluid in it was definitely light brownish yellow, but the colpidium was still purplish pink, though considerably darker, and the crystals were still attached to it and intact, with no change in color.

The fact that the neutral red crystals in the food vacuoles did not dissolve or change in color, whereas they became pink and dissolved in Clark buffer, pH 5, and in Hahnert solution, pH 4.5, indicates that the solution in them did not become more acid than pH 4.5. This and the fact that the chilomonads and the colpidia in the food vacuoles became purple (pH 3.5), i.e., much more acid than the fluid around them, indicate that the acid in the food organisms in the food vacuoles in *Amoeba* originates within the vacuoles, not in the cytoplasm around them. The fact that the fluid in the vacuoles becomes brownish yellow and that this occurs when the chilomonads and the colpidia in it are still pink, indicates that this fluid becomes alkaline and that the base in the fluid originates in the cytoplasm around the vacuole, not in the organisms in it. The results obtained in these observations therefore support the conclusions reached in the preceding experiments, and they indicate that the fluid in the food vacuoles does not become more acid than pH 4.5.

Further evidence concerning some of these conclusions was obtained by feeding the amoebae dead organisms.

Dead organisms ingested

Chilomonads and colpidia were respectively put into Hahnert solution containing neutral red and left about one hour, i.e., until the food vacuole in the latter and the neutral red bodies in the former were deeply stained. Then they were killed by rapidly heating the solution, after which enough NaOH was added to make the solution distinctly alkaline and the chilomonads and colpidia brownish yellow. Then

one drop of Hahnert solution containing *Amoeba dubia* was mounted on a slide and one drop of the solution containing the dead, stained chilomonads added, and the preparation observed for several hours. This was repeated with colpidia in place of chilomonads and also with *Amoeba proteus* in place of *Amoeba dubia*. The hydrogen ion concentration of the solution on the slide was measured with glass electrodes in every test. It varied from pH 7.4 to pH 7.8, and the chilomonads and colpidia in it from reddish yellow to brownish yellow. Some of the preparations contained neutral red crystals.

The amoeba ingested the dead chilomonads and colpidia readily, *Amoeba dubia* more readily than *Amoeba proteus*. The changes in the chilomonads and the colpidia in the food vacuoles were essentially the same in both species but they were somewhat more definite in the former than in the latter. After the yellow organisms had been ingested they gradually became pinkish, and in about 15 minutes they were pinkish red, about like neutral red in buffer, pH 7.4 or 7.2. They did not become purplish (pH 3.5 to 4) as they do when they die in solutions containing neutral red. During this time the fluid around the organisms was colorless but shortly became yellow. Then it and the organisms in it gradually became brownish yellow, like buffer pH 8 to 8.8. Then there was no further change in color in either for several hours, after which the color in both usually faded out gradually.

All these characteristics are sometimes seen simultaneously in different vacuoles in an amoeba. This is well brought out in the following description of the food vacuoles seen under high power (20 ocular and 60 objective, oil) in an amoeba which had been in the solution containing stained, dead chilomonads, a little more than three hours. During this time the amoeba had ingested 20 chilomonads. Some of these had much fluid around them, others had very little. In some this fluid was colorless, in others pinkish red (pH 7.2), in still others brownish yellow (pH 8.6). In some the color of the fluid was dense, in others very faint (barely visible). In some vacuoles the color of the chilomonads was the same as that of those in the culture fluid outside; in others it was pinkish red (about pH 7.2); in others yellow to yellowish brown (pH 8 to 8.8).

A considerable number of food vacuoles studied contained neutral red crystals. No changes were observed in any of these crystals. Some of the chilomonads and some of the colpidia studied were digested.

The fact that the neutral red stained chilomonads and colpidia were yellow (pH 7.4 to 7.8) when they were ingested and became pinkish red (pH 7.4 to 7.2) shows that the acidity of the chilomonads increased in the food vacuoles; and the fact that the chilomonads were

dead when they were ingested shows that this change in color was not due to chemical processes involved in metabolism or in dying. The results obtained, therefore, seem to indicate that acid was formed in the cytoplasm and poured into the vacuoles. It is evident, however, that the observed decrease in hydrogen ion concentration could have been due to addition of a weak alkaline solution. All that these results actually show, then, is that a solution which was not more alkaline than approximately pH 7.3 entered the food vacuoles.

Results which are in some respects similar to those presented above were obtained with starch grains in place of dead chilomonads and colpidia. These are presented below.

Starch ingested

Specimens of *Amoeba dubia* which had been without food 24 hours were mounted on slides, in Hahnert solution, containing neutral red and, respectively, rice, wheat and potato starch grains, and examined under low and high magnifications continuously for about 30 minutes and for short periods from time to time for 24 hours or longer. The wheat and rice starch used was taken from the bottom of dishes containing amoebae cultures in which wheat and rice grains had disintegrated. The potato starch was obtained by scraping a piece of potato, mixing the scrapings with water, straining through cheese cloth and washing.

The amoebae ingested the starch grains freely and some soon became well filled with them (Fig. 2). No changes were observed in the ingested grains for about 15 minutes; then they became purple, first faintly and then strongly like buffer pH 2 or less, then very gradually reddish pink (pH 7) and finally brownish yellow (pH 8.5 to 8.7).

The changes in color did not vary with the kind of starch but the colors were definitely more distinct in the potato and rice starch than in the wheat starch and the time required for the changes in color was somewhat less in the former than in the latter, but this varied greatly in all. It required about 15 minutes for the starch grains to become purple, about two hours more to become pinkish and about 12 hours more to become yellowish.

Usually there was but little fluid ingested with the starch grains and much of this soon disappeared, so that by the time the grains had become purple there was often no fluid visible in the food vacuoles. This was especially evident in those which contained potato starch. But in all the vacuoles in which fluid was visible it was invariably colorless until the starch began to become pinkish; then the amount of fluid in the vacuoles increased greatly and as it increased it became

yellow, so that now the vacuoles contained pink starch grains in a distinctly yellow fluid. The starch grains soon became yellow however and later brownish yellow. This indicates that the fluid and the starch grains in the vacuoles became alkaline but that the fluid became alkaline before the starch grains in it.

The results presented seem to indicate that the hydrogen ion concentration of the content of the food vacuoles in *Amoeba* becomes ex-

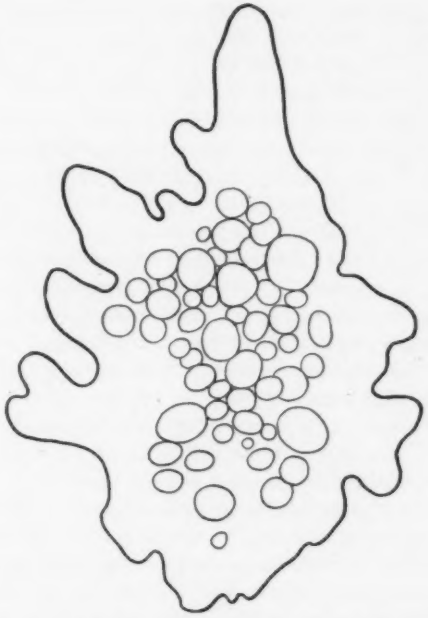

FIG. 2. Camera drawing showing *Amoeba dubia* after having ingested numerous potato starch grains.

traordinarily high, i.e., pH 2 or higher. The fact that neutral red crystals do not dissolve in the vacuoles, however, shows very clearly that it does not become nearly so high. What, then, is the cause of the purple color observed in the starch grains?

Amoebae which contained numerous neutral red stained starch grains varying in color from purple to brownish yellow were mounted in fresh neutral culture fluid, without any neutral red, and crushed by means of pressure on the cover-glass. Nearly all the starch grains flowed out and soon became free in the neutral fluid. Some of these

were crushed by further pressure, so as to expose the interior. The preparation was then carefully studied under 20 ocular and 60 oil immersion objective.

Observations on the crushed starch grains showed very clearly that they were colored throughout (i.e. that the color of the starch grains was not due to accumulation of dye on the surface, but to dye uniformly distributed through them) and they showed that the color in all the starch grains in the neutral fluid gradually faded until it had disappeared entirely, but that before the starch grains became colorless they invariably became purple, i.e. that those which were pink (pH 7) became purple (pH 2) then colorless and that those which were yellow (pH 8) became pink, then purple, then colorless.

The change from yellow (pH 8) to pink (pH 7) was doubtless due to increase in hydrogen ion concentration in the starch grains caused by the neutral fluid around them, but the change from pink to purple obviously could not have been due to this. It was in all probability correlated with the concentration of neutral red in the starch grains for it was repeatedly observed in neutral red solution that starch grains put into them always first became purple regardless of the hydrogen ion concentration, and it was also observed that in very weak solutions the purple color persisted much longer than in stronger solutions. This indicates that the structure of the starch grains is involved in the color, so that if the grain contains only a small amount of neutral red the light which passes through it is purple regardless of the hydrogen ion concentration. Neutral red is consequently under these circumstances not a reliable indicator in the acid range. Results presented below indicate that it is also unreliable as an indicator in the alkaline range.

In the experiments with neutral red it was repeatedly observed that the starch in the solution outside of the amoebae never became colored in the least. The question then arises as to what caused it to take up neutral red in the food vacuoles.

In attempting to answer this question, the following substances in various concentrations were added respectively to Hahnert solution (in small test tubes containing potato starch and neutral red in various concentrations) and left 24 hours or longer: sodium hydrate; hydrochloric, butyric, caprylic, capric, stearic, lactic and oleic acids; fresh, rancid and partially saponified olive oil; tripsin, pepsin and saliva; and lecithin and egg albumin. It was found that none of these substances had any effect on the absorption of neutral red by the starch except oleic and stearic acids and rancid and partially saponified olive oil. Of these, oleic acid and rancid olive oil were most effective but in the solutions containing these, even in very high concentration, the starch

never stained as densely as it did in the food vacuoles. If, however, oleic acid was added to dry starch and the starch then put into Hahnert solution containing neutral red, the starch stained just as deeply as it did in the food facuoles, and this also obtained for all the other acids tested. It would appear then that if starch contains acid it will take up neutral red from an aqueous solution but that oleic acid is the only one of those tested which is capable of replacing water in starch. The action of stearic acid and rancid and partially saponified olive oil is in all probability due to the presence of oleic acid in them.

What then is there in the food vacuoles in *Amoeba* which causes starch saturated with water to take up neutral red? The food vacuoles in *Amoeba*, as is well known, are surrounded by much plasmalemma which is ingested during their formation. This in all probability contains fatty acids which are similar to oleic acid and which, owing to decomposition, go into solution in the fluid in the food vacuole and become considerably concentrated as water in the vacuole is eliminated during the first 15 minutes after ingestion. It is probable that these concentrated fatty acids replace the water in the starch grains and cause them to take up neutral red.

This contention is supported by the fact that starch does not stain with neutral red in the food vacuoles in ciliates. Numerous observations were made in reference to this on *Paramecium caudatum*, *Vorticella similis* and *Colpidium striatum* in solutions containing neutral red and starch. All these ciliates ingested grains of potato and wheat starch very readily and frequently retained them as long as 30 minutes, but none of the starch grains became colored. In some instances, especially in the paramecia, the starch grains in the food vacuoles appeared to be definitely pink, but it was found that if pressure was applied so as to force the grains out, they invariably were colorless. The apparent color in them in the vacuoles was obviously due to numerous red granules in the cytoplasm adjoining the surface of the food vacuoles.

The food vacuoles in the ciliates, as is well known, are not surrounded by a membrane from the surface of the cell (a fatty acid-containing membrane) which decomposes, as in *Amoeba*, hence the lack of fatty acid to replace the water in the starch grains and the inability to absorb neutral red.

The results obtained in observations with neutral red seem to show that the fluid in the food vacuole in *Amoeba* becomes acid during the first 15 minutes after it has been ingested, that the acid probably originates in part from decomposition of some of the plasmalemma taken in during the process of ingestion and is correlated with elimination of fluid from the vacuole, that the content of the vacuole later

becomes alkaline and that this is correlated with entrance of fluid from the cytoplasm and marked increase in the size of the vacuole. These results however give no reliable information concerning the extent of either the acidity or the alkalinity. The following observations have a definite bearing on this problem.

The Maximum Acidity of the Fluid in the Food Vacuoles and Some Observations on Length of Life and Fission of Ingested Organisms

Congo red and brom cresol purple

Active yeast cells and chilomonads in Hahnert solution (pH 6.5) were killed by rapidly increasing the temperature, then congo red was added until the solution was saturated, after which the temperature was raised to boiling and held until the yeast cells and the chilomonads became brilliant orange in color. Amoebae which had been without food for 24 hours or longer were now mounted in Hahnert solution on slides and a little of the congo red solution containing the stained yeast cells and chilomonads added. Living chilomonads were also added to some of the preparations.

The amoebae ingested the yeast cells and the chilomonads freely but *Amoebia dubia* much more freely than *Amoeba proteus*. Some of the food vacuoles formed contained only yeast cells, others only dead chilomonads, others only living chilomonads, and others various combinations of these organisms in reference to number as well as kind. Many of these vacuoles in various amoebae were continuously observed under low and high magnification for an hour and briefly from time to time for 24 hours. No change in color was observed in the stained yeast cells or the stained chilomonads in any of them (no matter if they were alone or in combination with living chilomonads). They remained brilliant orange in color throughout the entire time of observation.[4]

Some of the stained yeast cells were now mounted in buffer solutions containing congo red. Those in buffer, pH 4.5 or higher, remained brilliant orange. Those in buffer, pH 4, became distinctly bluish and those in buffer, pH 3.5 and lower, intensely blue.

These results demonstrate therefore that the acidity of the solution in the food vacuoles in *Amoeba* does not become so high as pH 4, and

[4] Some of the preparations studied contained small amoebae (probably *Amoeba dofleini*) and paramecia. Stained yeast cells were ingested by both. In the former they remained orange, in the latter they became blue, remained so for a short time, then became orange again.

they support the conclusion reached in observations on neutral red crystals, but they do not show the maximum acidity reached.

The methods used in making observations with brom cresol purple were the same as those used in making observations with congo red. The yeast cells became densely sky blue in the hot brom cresol purple solution but the chilomonads did not stain well and were therefore used in only a few of the observations.

The yeast cells in the food vacuoles either alone or with living chilomonads remained sky blue usually for about 15 minutes after they had been ingested, then they gradually became greenish blue and finally greenish yellow, about like buffer, pH 5.6, containing brom cresol purple. Then no further change in color could be observed for some 12 hours, after which they gradually became sky blue again and remained so until they were ejected. There was no indication of any digestive action on the ejected yeast cells.

Stained yeast cells were mounted in buffer solutions containing brom cresol purple and observed under low and high magnification. The yeast cells in buffer, pH 6.4, or over became sky blue, those in buffer, pH 5.4 to 5.8, greenish yellow and those in buffer, pH 5 or less, distinctly yellow, but it was impossible to distinguish between those in any two of the buffers which differed by less than 0.4 pH units, e.g. those in buffer, pH 5.6, could be distinguished from those in buffer, pH 6 or pH 5.2, but not from those in buffer, pH 5.8 or pH 5.4.[5]

The fact, therefore, that the yeast cells in the vacuoles changed in color from sky blue to greenish yellow, strongly indicates that the acidity of the solution in the food vacuoles increased from that of the culture solution to about pH 5.6.

The time required for the changes in the color of the yeast cells in the food vacuoles varied very greatly. In one vacuole observed with two yeast cells and one chilomonad it required three minutes for the yeast cells to become greenish yellow; in another with one yeast cell and one chilomonad it required seven minutes; in another with four yeast cells and no chilomonads, nine minutes; in another with about 20 yeast cells, 35 minutes; and in another with 15 yeast cells and no

[5] Amoebae, chilomonads, paramecia and other organisms thrive for days in extraordinarily strong solutions of brom cresol purple in Hahnert solution, pH 6.5. The cytoplasm in the amoebae becomes distinctly yellowish green but the nucleus remains gray. However, if they die the nucleus becomes strongly blue and the cytoplasm faintly blue. The cause of these changes in color is not clear. The paramecia become very quiet. They ingest quantities of the stained yeast cells. These sky-blue cells (pH 6.5) become distinctly yellow a few seconds after the food vacuoles have left the gullet but later turn to blue again and remain so until they are eliminated. They are not digested.

chilomonads, 45 minutes, but in a large majority it required around 15 minutes. In general, it required longer for the change in color in vacuoles which contained many yeast cells than in those which contained but few, and longer in those which were near the surface than in those which were not. The presence of living chilomonads probably also has some effect although this was not definitely established.

The change from blue to greenish yellow in the yeast cells in the vacuole occurs after it has decreased to minimum in size and the later change to blue again after it has increased considerably. These changes are therefore correlated with changes in the amount of fluid in the vacuoles. The time required to kill the chilomonads after they were ingested varied greatly, but when they died the dead yeast cells ingested with them were almost always still sky blue. This shows that at the time when the chilomonads in the vacuoles die the acidity of the fluid around them is lower than pH 5.6, and consequently that their death is not specifically correlated with acidity for the lethal concentration is much higher than this, i.e. around pH 3.8, as will be demonstrated later.

Some interesting results obtained in observations made on three vacuoles follow:

(1) Several amoebae were mounted (4 P.M.) in Hahnert solution containing chilomonads, stained yeast cells and brom cresol purple, then observed almost continuously under an oil immersion objective.

(4:05 P.M.) An unusually large vacuole forming; contains 15 yeast cells, all sky blue, no chilomonads. (4:25 P.M.) Vacuole closed; amoeba very active, vacuole at the posterior end, no change in color. (4:50 P.M.) Vacuole much smaller, yeast cells slightly greenish. (5:00 P.M.) Yeast cells greenish yellow about pH 5.6. Vacuole continuously near the surface at the posterior end. (5:45 P.M.) No change. (6:00 P.M.) Vacuole broken up into four smaller ones; two with five yeast cells each, one with three and one with two; all near the center of the amoeba moving with the plasmasol; no change in color. (7:00 and 8:00 P.M.) No change. (10:00 P.M.) Yeast cells in the two smaller vacuoles distinctly bluish; one of the larger not found, no change in color in the other. (9:00 A.M.) Two vacuoles found, yeast cells in them sky blue.

(2) Amoebae mounted (3:50 P.M.) in Hahnert solution containing chilomonads, stained yeast cells and brom cresol purple.

(4:05 P.M.) A large vacuole with two yeast cells and one chilomonad formed; chilomonad very active, almost continuously under observation under oil immersion objective; yeast cells sky blue. (4:09

P.M.) Chilomonad very active; yeast cells sky blue. (4:13 P.M.) Chilomonad still active, no appreciable change in size of vacuole, yeast cells distinctly greenish yellow; the vacuole now very near the surface at the posterior end and its content very distinctly visible. During the following 30 minutes the vacuole remained very near the surface of the amoeba, it did not appreciably change in size, there was no visible change in the color of the yeast cells and the chilomonad was almost continuously very active. The chilomonad then suddenly came to rest, and a slight groove around it lengthwise was seen. This rapidly became deeper and in 45 seconds the chilomonad divided.[6] Both daughters soon became active and continued so for 50 minutes, then both suddenly died.

(3) Amoebae were mounted (4:30 P.M.) in Hahnert solution containing chilomonads, stained yeast cells and brom cresol purple and observed intermittently under oil immersion objective.

(4:50 P.M.) An amoeba found with one food vacuole which contained one yeast cell and a living chilomonad and three which contained respectively one, ten and 16 yeast cells but no chilomonads. All the yeast cells in the vacuoles were sky blue. The amoeba was very active and appeared to be in excellent condition. (5:30 P.M.) The yeast cells in the vacuole which contained ten were distinctly greenish yellow, about pH 5.6, those in the other three vacuoles were still sky blue; the chilomonad was still alive, and the vacuole which contained it was still large and at the posterior end very near the surface. (6:00 P.M.) No change; the vacuole containing the chilomonad was still large and very near the surface and the chilomonad active. The chilomonad therefore lived in this vacuole more than an hour and ten minutes and the solution in it did not become more acid than about pH 6.4. (9:00 A.M.) Several vacuoles found containing yeast cells but probably not the ones described above. Some of these contained indigestible remnants of chilomonads and were obviously old. The yeast cells in these were sky blue.

The results obtained in observations with brom cresol purple on yeast cell in food vacuoles in *Amoeba* show then that during about the first 15 minutes after the food vacuoles are formed the hydrogen ion concentration of the fluid in them increases from that of the culture fluid to around pH 5.6 and then decreases, but they give no information as to the extent of the decrease. This is the subject of the following observations.

[6] We studied a great many food vacuoles containing living organisms, but this is the only one in which we observed fission.

The Maximum Alkalinity of the Fluid in the Vacuoles

Cresol red

Two methods were used in the observations made with cresol red. In one the amoebae were fed on living colpidia in a solution of cresol red, in the other they were fed on colpidia which had been killed in a solution of cresol red.

(1) Several specimens of *Amoeba dubia* were put into a weak solution of cresol red in culture solution containing numerous colpidia in a watch-glass and left three days. The amoebae multiplied and became extraordinarily large and well filled with crystals, many of which had globular central portions and two long spine-like projections, extending in opposite directions from the central portion. A few drops of the solution containing four amoebae and a number of colpidia were mounted on a slide and a drop of strong cresol red solution added. This preparation was then covered with a cover-glass and sealed with vaseline. The amoebae moved and fed normally and multiplied extensively.[7] They were examined under low and high magnification (20 ocular and 60 oil immersion objective) from time to time for four days.

During this time colpidia were repeatedly seen in the process of ingestion and in various stages of digestion. They were compared in color with colpidia which had been killed in hot water and mounted in buffer solutions, pH 6 to 8, containing cresol red.

The colpidia in the buffer solutions, pH 7.8 and 8, were purplish (not red); those in the buffer solutions, pH 6.4, 6.2 and 6, were yellow and those in buffer solutions, pH 6.6 to 7.6, were intermediate.

The living colpidia in the food vacuoles were invariably colorless. Those which had recently died in the vacuoles were bright lemon yellow, i.e. acid probably beyond the range of the indicator, but the fluid around them was colorless. Those in all the later stages of digestion were yellow or brownish yellow and the fluid around them was yellow. There was no indication of a purplish tint comparable to that in the colpidia in buffers, pH 7.8 or 8.

This experiment was repeated several times with different concentrations of cresol red. No indication of purplish color as distinct as that in the colpidia in buffer, pH 7.8, was seen in any of the food vacuoles.

These results indicate therefore that the content of the food vacuole in *Amoeba* does not become so alkaline as pH 7.8 and probably not so alkaline as pH 7.6.

[7] *Amoeba* can withstand surprisingly strong solutions of cresol red without any ill effects and in moderate concentrations it appears to be decidedly beneficial.

(2) Colpidia were put into a saturated solution of cresol red and left until they died. Some were then mounted on a slide in a weaker solution of cresol red in Hahnert solution and several specimens of *Amoeba dubia*, which had been without food for 24 hours, added. The dead colpidia were yellow. The amoebae ingested them freely. The following day the amoebae contained numerous food vacuoles with colpidia in various stages of digestion. The content of these vacuoles varied from yellow in the younger ones to brownish yellow in the older ones. There was no indication whatever of a purplish tint (pH 7.8) in the content of any of them. These results therefore support the conclusions reached in the preceding experiments.

Phenol red

Colpidia were put into a saturated solution of phenol red in Hahnert solution and left until they died. Some of them were then put into buffer solutions, pH 6 to 8, containing phenol red and some into a drop of weak solution of phenol red in Hahnert solution on a slide which contained 15 starved specimens of *Amoeba proteus*.

The chilomonads in buffer, pH 7.2 or less, were yellow, those in buffer, pH 8 were red, and those in buffers, pH 7.4, 7.6 and 7.8, were pinkish yellow or yellowish pink.

The amoebae on the slide were examined continuously for two hours and briefly from time to time for two days. During the first two hours nearly half of the amoebae ingested one or more colpidia and at the end of 24 hours several had vacuoles containing colpidia in all stages of digestion. The colpidia were yellow when they were ingested and the fluid around them colorless. They remained yellow and the fluid colorless for several hours (this time varied greatly) then the amount of fluid in the vacuole increased considerably and became distinctly pinkish (pH 7.3). The colpidia in it were however still yellow but they also soon became pinkish.

These observations were repeated several times. *Amoeba dubia* was used in some of these tests. Essentially the same results were obtained in all. Specimens were repeatedly found which at a given time had some vacuoles in which the colpidia in them were yellow and the fluid colorless, some in which they were yellow and the fluid pinkish and some in which both were pinkish, and all these vacuoles intermingled freely in the plasmasol and frequently came in contact with each other.

The results obtained with phenol red seem to show then that the content of the older food vacuoles in *Amoeba* is slightly alkaline (pH 7.3, possibly 7.4), that the fluid in the vacuoles becomes alkaline before

the solid bodies in it and that the alkalinity is correlated with increase in the amount of fluid in the vacuoles. And the results obtained with brom cresol purple seem to show that the fluid in the younger food vacuoles is somewhat acid, probably pH 5.6.

There, however, still remains the question as to whether or not the changes in the color of the content of the food vacuoles in *Amoeba* are specifically correlated with changes in hydrogen ion concentration; for it is well known that the oxygen tension in cytoplasm is very low and that many dyes reduce readily and change greatly in color. This matter will be discussed in some detail in a subsequent paper. Suffice it to say here that results obtained with reducing and oxidizing agents indicate that the color of the dyes in question is probably not appreciably, if at all, affected by the low oxygen tension in the cytoplasm.

The Origin of the Acid and the Base in the Food Vacuoles in *Amoeba*

It is widely held, as previously stated, that the cytoplasm in the protozoa secretes acid and base and "pours" them into the food vacuoles, the former when the vacuoles are young and the latter after they are old.

It is well known that in *Paramecium* and some other ciliates granules which stain deep red with neutral red aggregate at the surface of the food vacuoles. It has been suggested that these contain acid and that they pass into the food vacuole and cause increase in the acidity of its content. There are, however, no such granules in the cytoplasm of *Amoeba* and there is no indication of aggregation of granules of any kind at the surface of the food vacuoles in them. The increase in the acidity of the content of the food vacuoles, therefore, obviously cannot be correlated with granules in the cytoplasm. Moreover, the results presented above show that the increase in the acidity of the fluid in the food vacuoles in *Amoeba* occurs simultaneously with marked decrease in the size of the vacuole, i.e. that it occurs while there is a continuous flow of fluid from the vacuole out into the cytoplasm. It is very difficult to see how acid could, at this time, pass from the cytoplasm into the vacuole against the outward flow of fluid.

These considerations consequently strongly support the conclusion reached above, namely, that the acid which causes the increase in the acidity of the fluid in the food vacuoles in *Amoeba* originates within the vacuoles, not in the cytoplasm around them. But they do not illuminate the processes involved in the production of the acid within the vacuoles.

In the observations on ingested starch it was concluded that some of the acid in the vacuoles probably originates from the decomposition of the plasmalemma ingested during the formation of the vacuoles. Some is, however, produced by metabolism in living organisms in the vacuoles and by the processes involved in the death of these organisms, as is clearly indicated by the great changes observed in the color of absorbed indicators in them. Observations concerning the effect of metabolism on the hydrogen ion concentration of culture fluid follow:

Colpidia from a vigorous culture were concentrated in tap water by means of the centrifuge; then about 0.25 cc. added to each of three 5 cc. vials. The vials were then filled with balanced salt solution (0.001 M) and a little brom thymol blue added to one, brom phenol blue to another and brom cresol green to the third. The vials were then closed air-tight with paraffin impregnated cork stoppers, in such a way that all free air was eliminated. The color of the solution in the vials was then, at five minute intervals, compared with that of standard buffers and the hydrogen ion concentration ascertained. The following results were obtained:

Vials closed 9:20 A.M.; solution in them, pH 7.4; 9:25, pH 7; 9:30, pH 6.8; 9:35, pH 6.4; 9:40, pH 6; 9:45, pH 5.4; 9:50, pH 4.8; 10:00, pH 4.5 (about one-half of the colpidia were now inactive); 10:10, pH 4.4; 10:30, pH 4.4 (still many inactive); 11:30, pH 4.4; 3:30, pH 4.4 (still some active).

This experiment was repeated with colpidia and also with chilomonads and essentially the same results obtained. These results prove conclusively that metabolism in living colpidia and chilomonads in air-tight enclosures produces marked increase in the acidity of enclosed solutions. The rate of increase under these conditions varies, of course, directly with the volumetric ratio of organism to solution and with the activity of the organisms. In the food vacuoles in *Amoeba* this ratio is fairly large and the ingested organisms are extremely active. It is therefore likely that the metabolism of the organisms in the vacuole produces considerable increase in acidity in the fluid in the vacuoles.

There is also considerable evidence (which will be presented in a subsequent paper) that the membrane at the surface of the food vacuoles is not permeable to the acid produced in them. If this evidence is valid, it is obvious that the production of a very small amount of acid in the vacuoles shortly after they are formed would, owing to subsequent great loss of fluid, result in great increase in the acidity of the fluid which remains when the vacuole has decreased to minimum in size.

There is then in the results obtained in observations on the food vacuoles in *Amoeba* no support whatever for the contention that the cytoplasm at the surface of the younger food vacuoles secretes acid and pours it into the vacuoles.

It was demonstrated above that the increase in alkalinity of the content of the food vacuoles in *Amoeba* occurs simultaneously with increase in the size of the vacuoles and entrance of fluid from the adjoining cytoplasm, and that the fluid in the vacuoles becomes alkaline before the solid substance suspended in it. It was consequently concluded that the base which causes this fluid to become alkaline originates in the cytoplasm around the food vacuole. This does not show, however, that the base is secreted by the cytoplasm, for the alkalinity of the fluid, as demonstrated above, is only approximately pH 7.3, and it is highly probable that all the fluid in the cytoplasm is equally alkaline.

Functions of Acid and Base in the Food Vacuoles in *Amoeba*

A number of investigators, as previously stated, maintain that the acid in the food vacuoles in protozoa functions as a killing agent. It was demonstrated in the observations with brom cresol purple, described above, that the fluid in the food vacuoles in *Amoeba* does not become more acid than approximately pH 5.6. It is consequently evident that if the acid in these vacuoles functions as a killing agent this concentration must be lethal for the ingested organisms which die in the vacuoles, notably *Colpidium* and *Chilomonas*. The following experiments concern this problem:

(1) To Hahnert solution in a series of small flasks different quantities of HCl were added and the hydrogen ion concentration of the solution in each measured with glass electrodes. Then 0.1 cc. of each was put respectively into depressions on hollow ground slides and a very small quantity of solution containing numerous chilomonads added and thoroughly stirred. The preparations were then, at short intervals, examined under low and high magnification. The following results were obtained:

In solution, pH 3.5, the chilomonads died immediately; in solution, pH 3.9, about half of them died immediately and the rest in less than one minute; in solution, pH 4.1, about half of them became inactive in two minutes but many of these gradually recovered, so that after 15 minutes nearly all were active; in solution, pH 4.2, about one-fourth of them became inactive and practically all these soon became active again.

This experiment was repeated twice and essentially the same results were obtained. It was also repeated six times with colpidia in place of chilomonads. In three of these lactic acid was added in place of hydrochloric acid.

It was found that the colpidia lived more than four hours in lactic acid (pH 3.5) and that a few lived more than six hours in this acid (pH 3), although many died in less than two minutes. In hydrochloric acid the lethal concentration was slightly lower, but not quite so low as it was for chilomonads.

The results obtained indicate, therefore, that to kill either chilomonads or colpidia with acid, it requires a concentration of at least pH 4. It is consequently evident that since the acidity of the fluid in the food vacuoles in *Amoeba* does not exceed approximately pH 5.6, the death of these organisms in the vacuoles cannot be due to acid. Nirenstein (1905) comes to the same conclusion in reference to bacteria in the food vacuoles in paramecia. Moreover, when colpidia die in weak solutions of acids the contractile vacuole stops pulsating before the cilia stop beating but fluid continues to flow into it and it consequently becomes much enlarged. These phenomena do not occur when the colpidia die in the food vacuoles. Under these conditions the contractile vacuoles continue to pulsate for some time after the cilia stop beating and there is no indication whatever of any enlargement. These observations consequently support the conclusions that death of organisms in the food vacuoles is not caused by acid.

Nirenstein (1905) et al. maintain that digestion of organisms in the food vacuoles does not begin until after their content has become alkaline. The results of observations on *Amoeba* are in full accord with this contention. The evidence in hand seems, therefore, to indicate that the acid does not function in digestion. Nirenstein suggests, however, that it may function in activating "Profermente" and in coagulating proteins, so as to facilitate the action of triptase. This suggestion was probably the result of his conviction that the acid is secreted by the cytoplasm adjoining the food vacuoles and that it consequently must have some function. If, however, the analysis presented above concerning the origin of the acid is correct, there is obviously no need of postulating any function for it. Moreover, the fact that while the content of the vacuole is acid, there is a continuous outward flow of fluid, makes it difficult to see how any digestive enzymes could enter during this time. This fact, therefore, also militates against the view that the acid functions in digestion.

It was demonstrated above that after the acidity in the vacuole has become maximum, fluid rapidly passes from the cytoplasm into the

vacuole. This is doubtless due to increase in the osmotic concentration of the fluid in the vacuole, owing, at least in part, to diffusion from the dead organism in the vacuole; and it may well be that this diffusion and the consequent increase in osmotic concentration is facilitated by action of the acid on the organism. Moreover, the acid may also act on the diffused substance in such a way as to increase the osmotic concentration.

It is generally held that the base in the food vacuole functions in facilitating the action of enzymes on the ingested organisms.

As stated above, the change from acid to alkaline in the food vacuole coincides with a rapid increase in the size of the vacuole owing to rapid inflow of fluid from the cytoplasm. This fluid is doubtless alkaline and it probably contains enzymes which are carried into the vacuole where they act on the ingested organisms.

Factors Involved in the Changes in the Size of the Food Vacuole in *Amoeba*

As previously stated, the food vacuole in *Amoeba*, beginning immediately after it is formed, decreases greatly in size, then rapidly increases considerably and then very gradually decreases until it is eliminated (Fig. 1). The question now arises as to what causes these changes.

Very soon after the vacuole has been formed, sheets of cytoplasm usually pass through it separating off portions. This reduces the size of the vacuole. The fluid in the vacuole is, at this time, essentially like the culture fluid outside. The osmotic concentration of this fluid is lower than that of the fluid in the cytoplasm (Mast, 1923, 1926). There is consequently a flow of fluid out of the vacuole, resulting in reduction in size. After the size has reached minimum, the organisms in the vacuole die and shortly after this fluid passes rapidly from the cytoplasm into the vacuole causing rapid increase in size. This is doubtless due to sudden increase in the osmotic concentration of the fluid in the vacuole, owing to diffusion from the dead organisms in it. Digestion begins at this time and the subsequent gradual reduction in the size of the vacuole is doubtless due to diffusion out into the cytoplasm, of various substances formed during this process.

The Cause of Death of the Organisms in the Food Vacuoles

It was concluded above that death of the organisms in the food vacuoles in *Amoeba* is not due to acid in the vacuoles and it was noted that Nirenstein reached the same conclusion in reference to organisms in the food vacuoles in *Paramecium*. Nirenstein (1905) contends that

death in these vacuoles is caused by some other toxic substance which passes from the surrounding cytoplasm into them. This is, however, in all probability not true in *Amoeba*, for during the entire time between the formation of the food vacuole in this form and death of the organisms in it, there is a continuous flow of fluid out of the vacuoles. What then is it that kills the organisms?

It is well known that the oxygen tension is much lower in active cytoplasm than it is in culture fluid. There is consequently a flow of oxygen from the culture fluid in the vacuoles out into the cytoplasm. This doubtless results in marked reduction in the oxygen tension in this fluid. Moreover, the ingested organisms, as stated above, are extremely active and consequently consume much oxygen in respiration. This doubtless augments the reduction in oxygen tension in the vacuoles considerably. And, furthermore, the amount of fluid in the vacuoles decreases rapidly and this obviously increases the effect of respiration on the reduction in oxygen tension in the fluid remaining in the vacuole. The oxygen tension of this fluid is then doubtless soon reduced nearly, if not quite, to that in the cytoplasm around the vacuoles and it may well be that this is fatal to the ingested organisms. This conclusion is supported by the fact that death in the vacuoles is definitely correlated with reduction in their size (Fig. 1, 3), and by the fact, substantiated in the preceding section on brom cresol purple, that the length of life in the vacuoles is greatly increased if they are near the surface so that oxygen can diffuse into them from the surrounding culture fluid. The results obtained in the following observations on organisms enclosed in air-tight tubes also lend some support to the conclusion.

A number of capillary glass tubes about 2 cm. long and 0.5 mm. in diameter were drawn out to a diameter of about 0.25 mm. at either end. Solution from a vigorous culture of *Chilomonas* was centrifuged until the chilomonads were densely packed in the bottom of the tubes, then the free fluid was poured off. The substance which remained consisted of about four parts of culture fluid to one part of chilomonads. Two of the capillary tubes were entirely filled with this substance and the two ends of each sealed with melted paraffin. A little of the poured-off fluid was then returned to the centrifuge tubes and two more capillary tubes filled and sealed, then a little more fluid was added and two more filled and sealed. The chilomonads in each of the six tubes were then studied at short intervals. This was repeated with colpidia in place of chilomonads and with several more concentrations of organisms.

With chilomonads in the tubes containing ten parts of fluid to one part of organisms, a few died in five minutes and all in 33 minutes; in 40 to one concentration, none died in 20 minutes and all in four hours; in concentration 150 to one, none died in 50 minutes and all in four hours.

With colpidia in the tubes containing four parts of fluid to one part of organisms, a few colpidia died in 13 minutes and all in 47 minutes;

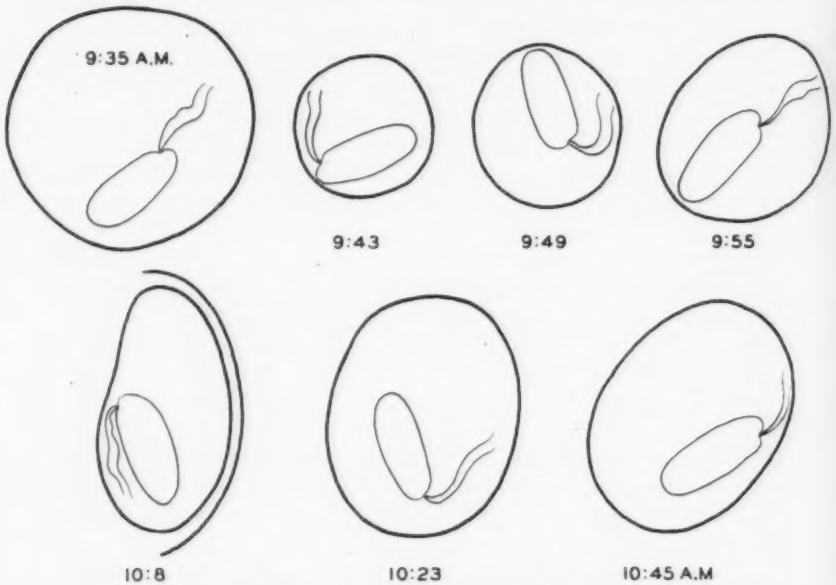

FIG. 3. Camera outlines showing changes in the size of a food vacuole in *Amoeba proteus*, containing an ingested chilomonad. The chilomonad was still active after having been in the vacuole for more than an hour.

Note that there was still much fluid in the vacuole when it had become minimum in size. Compare with Figure 1. This indicates that death of organisms in the food vacuoles is correlated with the extent of the reduction in the amount of fluid they contain.

in concentration six to one, a few died in 16 minutes and all in 55 minutes; in concentration ten to one, a few died in 20 minutes and all in 105 minutes; in concentration 15 to one, a few died in 100 minutes and all in 210 minutes.

These results demonstrate that organisms in fluid in an enclosed tube which simulates a food vacuole, rapidly produce a condition in the fluid (probably due mainly to reduction in oxygen tension) which

is fatal. It is consequently not necessary to postulate production of toxic substance by the surrounding cytoplasm to account for death in the food vacuoles.

Digestion in the Food Vacuoles in *Amoeba*

Greenwood (1886) maintains that proteins are digested in *Amoeba* but that starch is not and that digestion of fat is doubtful. Nirenstein (1905) says there is no evidence which indicates that fat is digested in any of the unicellular organisms. Dawson and Belkin (1928, 1929) conclude that if oil is injected into amoebae some kinds are digested and others are not. This conclusion has been confirmed by Wilber (1942) in observations on various oils injected into *Pelomyxa carolinensis*. Mast and Hahnert (1935) and Mast (1938) proved that the fat, the starch and the protein found in chilomonads are digested in the food vacuoles in *Amoeba*. Since this occurs at room temperature in a weak alkaline solution these food vacuoles must contain enzymes.

It is generally assumed that the enzymes found in the food vacuoles are formed in the cytoplasm of the amoebae and pass from it into the vacuoles. Mast and Doyle (1935) suggested that the beta granules (mitochondria) are involved in this transfer and others have postulated other carriers. There is, however, very little evidence in support of these suggestions. Moreover, there is no necessity for the postulation of special carriers, for, as stated above, much fluid passes from the cytoplasm into the vacuoles shortly before digestion begins and enzymes could be carried in with it. Furthermore, there is evidence which strongly indicates that at least some of the enzymes do not originate in the cytoplasm of the amoebae, and consequently are not carried into the food vacuoles.

Greenwood used wheat starch in her observation on digestion in *Amoeba* and found that the grains were often retained for several days without any indication whatever of digestion. I obtained similar results with potato and rice starch as well as with wheat starch. The starch grains were freely ingested. The food vacuoles changed in size normally and fluid passed from the cytoplasm into them. The starch grains were retained for hours and sometimes for days without the slightest indication of etching (digestion) on any of them. This seems to show that there was no amylase in these food vacuoles and none in the cytoplasm in the amoebae. How then can the digestion of the starch in chilomonads observed by Mast and Hahnert be explained?

Mast and Pace (1933) found that if chilomonads are starved, the starch in them soon disappears, owing to hydrolysis. This shows

that the chilomonads contain amylase. The food vacuoles which contain chilomonads therefore obviously also contain amylase, and the digestion of the starch in them is doubtless due to the action of this amylase. There is, therefore, no need for the postulation of amylase in the cytoplasm of amoebae to account for the digestion of the starch in the ingested chilomonads.

Holter and Doyle (1938) in some very accurately controlled experiments found considerable amylase in amoebae. If there is none in the cytoplasm of these organisms, how can this be explained? In their experiments they took about 1000 amoebae which had been feeding on chilomonads, reduced them to a "homogeneous suspension in M/120 phosphate buffer" and found amylase in this suspension. The amoebae used doubtless contained chilomonads in food vacuoles. It is consequently obvious that the amylase found may have had its origin in these chilomonads and not in the cytoplasm. The results obtained by Holter and Doyle do not, therefore, militate against the contention that the cytoplasm of *Amoeba* contains no amylase.

The fact that fat injected into *Amoeba* is digested seems to show that the cytoplasm contains lipase, but at least some of that found in the food vacuoles probably also originates in the ingested organisms. And this can also be said concerning the origin of the pepdidase found there.

The Hydrogen Ion Concentration of the Fluid in the Cytoplasm in *Amoeba*

Pantin (1923) maintains that the hydrogen ion concentration of the cytoplasm in a small marine amoeba is pH 7.6–7.8 in the plasmasol, pH 7.2 in the plasmagel and pH 6.8 in the protruding pseudopods. Needham and Needham (1925) conclude that in *Amoeba proteus* it is pH 7.6 throughout, and Chambers, Pollack and Hiller (1927) contend that in *Amoeba proteus* and *Amoeba dubia* it is pH 6.9 $\pm$ 0.1.

The results presented above show that the maximum alkalinity of the fluid in the food vacuoles in *Amoeba proteus* is approximately pH 7.3 and that this obtains immediately after a relatively large quantity of fluid has passed from the cytoplasm into the vacuole. They show that before this the fluid in it is slightly acid. The fluid which passes in from the cytoplasm must therefore be somewhat more alkaline than pH 7.3. The quantity that is in the vacuole at this time is however so small compared with the quantity which enters, that the effect of the former on the alkalinity of the latter must be slight. The fluid in the cytoplasm is therefore probably approximately pH 7.4, unless that which passes from the cytoplasm into the food vacuoles differs in

acidity from that which is ordinarily found in it. This, however, is not at all likely. Moreover, it has been known for some time that the fluid in the crystal vacuoles in *Amoeba* is definitely alkaline (Mast 1923, 1926). This supports the conclusion that the fluid in the cytoplasm is alkaline.

The conclusion of Chambers et al. that the acidity of the cytoplasm in *Amoeba* is pH 6.9 $\pm$ 0.1 is based upon changes in color of several different injected dyes. It is well known that injury causes marked increase in the acidity of the cytoplasm and it is, obviously, impossible to inject dyes without injuring the cytoplasm. It is, therefore, highly probable that the values obtained by Chambers et al. are somewhat too low. Their results therefore do not militate against the contention that the hydrogen ion concentration of the fluid in the cytoplasm in general is the same as that of the fluid which passes from the cytoplasm into the food vacuoles, i.e. that it is approximately pH 7.4.

Pantin's conclusion, stated above, is based upon observations on marine amoebae stained with neutral red. He does not say what it is that stains in these amoebae. In *Amoeba proteus* staining with neutral red is confined to various substances in the food vacuoles, the refractive bodies and the fluid in the vacuoles which contain crystals. The refractive bodies become crimson. They are clearly acid in reaction, owing doubtless to the presence of fatty acids. The fluid in the crystal vacuoles becomes brownish yellow, showing that it is definitely alkaline in reaction. The content of some of the food vacuoles is distinctly acid, that of others distinctly alkaline, and that of still others partly acid and partly alkaline. There are, then, in the cytoplasm of *Amoeba proteus*, at any given time localized acid and alkaline substances, and in specimens which are stained with neutral red, localized red and yellow regions. Under moderate magnification these colored regions make the entire cytoplasm appear to be stained either red or yellow or intermediate, depending upon the relative number of red and yellow regions. I have found that if observations are made with a first class optical system containing an oil immersion objective there is no indication of any color in the cytoplasm between the localized colored regions. The color seen in these regions under moderate magnification is doubtless due to the action of the colored regions on the light which passes through the cytoplasm. It is consequently obvious that the conclusions based upon the appearance under moderate magnification of different regions in an amoeba stained with neutral red are not reliable.

Pantin does not describe the optical system used, but his statements imply that the amoebae studied contained numerous granules in the form of a network and that these granules stained with neutral red.

It may well be, then, that all the color observed originated in these granules and that the different shades depended upon their concentration and distribution, and not upon the hydrogen ion concentration of the fluid in the cytoplasm *per se*.

Needham and Needham base their conclusions concerning the hydrogen ion concentration of the cytoplasm of *Amoeba* on observations made under moderate magnification ("$\frac{1}{4}$ inch objective and a Watson eyepiece giving a magnification of 15") on some specimens stained by immersion in a solution of neutral red, and others injected respectively with neutral red, brom thymol blue and phenol red. They say they used *Amoeba proteus*, but give no description. They maintain that the cytoplasm in all the tests assumed a color like that of Clark buffer, pH 7.6.

If they actually had *Amoeba proteus*, the color observed in the cytoplasm of specimens stained by immersion in a solution of neutral red was undoubtedly due to the color of the content of the food vacuoles, the refractive bodies and the fluid in the crystal vacuoles, and not to color in the fluid in the cytoplasm. Whether or not this obtains for injected specimens is not known, but it does throw considerable doubt on the validity of the conclusions reached, namely, that the hydrogen ion concentration of the cytoplasm in *Amoeba proteus* is pH 7.6.

Summary

1. In *Amoeba* new food vacuoles which contain chilomonads or colpidia decrease greatly in size and the organisms in them die soon after they have decreased to minimum; then the size increases rapidly and digestion begins, after which the size decreases very gradually.

2. If the culture fluid contains neutral red, the organisms in the vacuoles become purple (like buffer solution, pH 3.5, containing neutral red) immediately after they die, but the fluid around them remains colorless until after the vacuoles have increased in size, then it becomes yellow like buffer, pH 8, but the organisms in it remain purple for some time after this, then become brownish yellow.

3. The color of the organisms immediately after death in the food vacuoles is the same as that of organisms which have died in a solution of neutral red outside. This and the fact that the fluid in the food vacuole does not, at this time, become colored indicates that the acid originates in the vacuole, not in the cytoplasm around it. The fact that the fluid in the vacuole later becomes yellow before the organisms in it do, indicates that the base originates in the cytoplasm around the vacuole, not in the organisms in it.

4. Potato or wheat starch grains ingested by amoebae in culture fluid containing neutral red become purple like buffer, pH 2. This color is, however, largely a physical phenomenon, and therefore does not accurately indicate hydrogen ion concentration.

5. Neutral red crystals become pink and dissolve in buffer, pH 5, but they do not become pink and do not dissolve in the solution in the food vacuoles, either if they are alone or if they are in the vacuoles with organisms. This solution, therefore, does not become as strongly acid as pH 5.

6. Chilomonads killed in alkaline solution containing congo red are brilliant orange in color. They become bluish in buffer, pH 4 to 3.5. Amoebae ingest them readily. They remain orange in color in the food vacuoles. This shows that the acidity of the fluid in the vacuoles does not reach pH 4.

7. Chilomonads killed in alkaline solution of brom cresol purple are strongly purple. They become yellowish in buffer solution, pH 5.8 to 5.6. In the food vacuole they become slightly yellowish. This indicates that the acidity of the solution in the vacuoles reaches at least pH 5.6.

8. Results obtained with chilomonads killed respectively in cresol red and phenol red indicate that the maximum alkalinity of fluid in the food vacuoles is approximately pH 7.3.

9. The hydrogen ion concentration in the food vacuoles in *Amoeba* changes from about pH 5.6 to about pH 7.3, i.e. not nearly so much as indicated by the changes in the color of the content of food vacuoles which contain neutral red.

10. The increase in the acidity of the fluid in the food vacuoles probably is due to respiration in the ingested organisms, chemical changes associated with their death, disintegration of the ingested plasmalemma, impermeability to acids of the membrane around the vacuoles and diffusion of fluid from the vacuoles. The decrease in acidity is due to diffusion of alkaline fluid from the cytoplasm into the vacuoles. The cytoplasm secretes neither acid nor base.

11. The acid in the food vacuoles probably facilitates increase in the osmotic concentration of the fluid in the vacuole, resulting in diffusion of fluid containing enzymes from the cytoplasm into it. The base promotes digestion.

12. Death of the organisms in the food vacuoles is not caused by acid. It probably is caused by decrease in oxygen in the vacuoles, owing to respiration of the organisms in them, diffusion of oxygen out into the cytoplasm and decrease in the volume of fluid in the vacuoles.

13. The changes in the size of the food vacuoles are probably due to differences between internal and external osmotic concentrations.

14. The hydrogen ion concentration of the fluid in the cytoplasm is approximately pH 7.4.

LITERATURE CITED

CHAMBERS, R., POLLACK, H., AND HILLER, S., 1927. The protoplasmic pH of living cells. *Proc. Soc. Exp. Biol. and Med.*, **24**: 760–761.

CLAFF, C. L., DEWEY, VIRGINIA C., AND KIDDER, G. W., 1941. Feeding mechanisms and nutrition in three species of Bresslaua. *Biol. Bull.*, **81**: 221–234.

DAWSON, J. A. AND BELKIN, M., 1928. The digestion of oils by *Amoeba dubia*. *Proc. Soc. Exp. Biol. and Med.*, **25**: 790–793.

DAWSON, J. A., AND BELKIN, M., 1929. The digestion of oils by *Amoeba proteus*. *Biol. Bull.*, **56**: 80–86.

GREENWOOD, M., 1886. On the digestive processes in some rhizopods. *Jour. Physiol.*, **7**: 253–273; **8**: 263–287.

GREENWOOD, M., AND SAUNDERS, E. R., 1894. On the role of acid in protozoan digestion. *Jour. Physiol.*, **14**: 441–467.

HAHNERT, W. F., 1932. Studies on the chemical needs of *Amoeba proteus*. *Biol. Bull.*, **62**: 205–211.

HEMMETER, J. C., 1896. On the role of acid in the digestion of certain rhizopods. *Amer. Nat.*, **30**: 619–625.

HOLTER, H. AND DOYLE, W. L., 1938. Studies on enzymatic histochemistry. XXVIII. Enzymatic studies on protozoa. *Jour. Cell. and Comp. Physiol.*, **12**: 295–308.

HOWLAND, RUTH B., 1928. The pH of gastric vacuoles. *Protoplasma*, **5**: 127–134.

MAST, S. O., 1923. Mechanics of locomotion in *Amoeba*. *Proc. Nat. Acad. Sci.*, **9**: 258–261.

MAST, S. O., 1926. Structure, movement, locomotion and stimulation in *Amoeba*. *Jour. Morph. and Physiol.*, **41**: 347–425.

MAST S. O., 1938. Digestion of fat in *Amoeba proteus*. *Biol. Bull.*, **75**: 389–394.

MAST, S. O., AND DOYLE, W. L., 1935. Structure, origin and function of cytoplasmic constituents in *Amoeba proteus*. II. Origin and function based on experimental evidence; effect of centrifuging on *Amoeba proteus*. *Arch. f. Protistk.*, **86**: 278–306.

MAST, S. O., AND HAHNERT, W. F., 1935. Feeding, digestion and starvation in *Amoeba proteus* (Leidy). *Physiol. Zool.*, **8**: 255–272.

MAST S. O., AND PACE, D. M., 1933. Synthesis from inorganic compounds of starch, fats, proteins and protoplasm in the colorless animal *Chilomonas paramecium*. *Protoplasma*, **20**: 326–358.

NEEDHAM, J. AND NEEDHAM, DOROTHY M., 1925. The hydrogen ion concentration and the oxidation-reduction potential of the cell-interior. *Proc. Roy. Soc. London*, Ser. B, **98**: 259–286.

NIRENSTEIN, E., 1905. Beiträge zur Ernährungsphysiologie der Protisten. *Zeitsch. f. allg. Physiol.*, **5**: 435–510.

PANTIN, C. F. A., 1923. On the physiology of amoeboid movement. I. *Jour. Mar. Biol. Assoc.*, **13**: 24–69.

WILBER, C. G., 1942. Origin and function of cytoplasmic constituents in *Pelomyxa carolinensis*. Dissertation. Johns Hopkins University Library.

A SEROLOGICAL STUDY OF SOME AVIAN RELATIONSHIPS[1]

RALPH J. DEFALCO

(From the Department of Zoology, Rutgers University, New Brunswick, New Jersey)

INTRODUCTION

Serological techniques have played and will continue to play an important role in the construction of a better system of classification. As an independent, and more objective, source of information, serological tests have in general corroborated the broad outlines of animal classification.

Boyden (1942) in an extensive survey of the literature on systematic serology, in which is embodied a critical analysis of the theory and fact of systematic serology, clearly indicates the complementary nature of serological data to morphologically established facts. There are, however, certain instances in which the systematic position of the forms concerned is admittedly "temporary" or purely speculative. This is either the result of conflicting views among morphologists, or disagreement between adult anatomical and developmental data. Such conflicts are bound to occur when the data are largely subjective. A recent publication by Wilhelmi (1942) demonstrates how well the relatively objective precipitin reaction lends itself to the "testing" of theories concerning the origin of vertebrates. Confirming the belief of some zoologists, Wilhelmi finds that the relationship between the echinoderms and prochordates is far greater than that indicated between annelids or arthropods and prochordates. There is good reason to believe, therefore, that serological techniques may eventually give us the answer to the "phyla of uncertain systematic position." The selection of the proper antigens (tissues, fluids, etc.) to be compared will play the important role in such investigations.

Most of the serological phylogenetic information thus far has come to us through the use of the ring test. In this test, when properly performed, comparable dilutions of several antigens are permitted to react with a constant amount of an antiserum made to one of the antigens. The highest dilution of each antigen giving a reaction with the antiserum is noted. These end-points are compared and the degree of

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similarity of the antigens is evaluated on the basis of the amount of antigen needed to produce the "final ring." Actually only one point, however, in the entire reaction is compared (Boyden, 1942). Other methods, Boyden and Baier (1929), Baier (1933), Wolfe and Baier (1938) and Baier and Wolfe (1942), have been employed to compare antigens and determine relationships; most of these are more sound, statistically, because of the relatively smaller errors involved. These investigators have recorded the volume of precipitate formed when several dilutions of the antigen were tested with homologous and heterologous antisera. This procedure has enabled them to differentiate between the sera of very closely related forms.

The invention of the photronreflectometer, a photoelectric nephelometer, by Libby (1938), and its first application in relationship studies (crustacean and mammalian) by Boyden (1939) have served as the impetus for the extension of a problem which had been confined to ring test technique. This instrument has provided the means of procuring a rapid, accurate, and more nearly quantitative determination of the degree of chemical similarity of antigens than is made possible by the widely used ring test method. The successful use of the instrument with such heterogeneous groups as crustaceans and mammals made it desirable to test the instrument with a homogeneous group such as birds. It was also considered important to compare the values obtained by photronreflectometric tests with those obtained by ring test technique as previously suggested by DeFalco (1941).

Erhardt's (1930) tabulated survey of the literature and the results of his own researches indicate the possibility of distinguishing some of the birds from others. The experiments of Sasaki (1928) have demonstrated that it is also possible to distinguish between the species of the same genus; and those of Cumley and Irwin (1940) show that it is possible to distinguish between the serum of a hybrid dove and that of its parents. Some of the work by other investigators (Graham-Smith, 1904; Cohen, 1939) has made it possible to link the birds with the reptiles (Crocodilia and Chelonia) with considerable success.

Materials and Methods

While the tables and figures which follow indicate the use of one sample of antigen in each test, actually each sample represents the pooled sera of from four to 95 birds, with the exception of turkey buzzard which is the serum from one individual. These samples are probably typical of the species they represent because duplicate and triplicate pooled samples (with the one exception noted above) from entirely different sources were tested against a common antiserum

before one was chosen for this series of experiments. In all cases the birds of the same breed were killed at one time and their bloods permitted to clot in a common container. After a period of from seven to 15 hours the expressed serum was centrifuged until free of cells, then filtered sterile with a Seitz filter.

The crystalline egg albumins of leghorn, turkey, duck, and goose were prepared according to the method of Hopkins and Pinkus (1898). Each albumin was precipitated three times to free it from as much of the other proteins as possible.

The hemoglobins were prepared by the method of Welker and Williamson (1920). Spectroscopic examination by Dr. James B. Allison, of the Department of Physiology and Biochemistry, Rutgers University, revealed that these antigens were in the methemoglobin form.

The lens proteins were prepared by grinding clean, washed lenses in a mortar. The resulting pasty material was then dissolved in buffered saline, filtered sterile, and preserved in the refrigerator as were all other antigens.

To study the possible effect of the lipids on the serological reactions of antigens, a portion of each antigen was extracted with petroleum-ether (Merck's Benzin B. P. 30°–60° C.) for a period of 24 hours. The antigens were extracted in a Friedrichs extracting chamber in which ether, condensing, drops to the bottom of a side arm tube containing the antigen. As the ether collects on the surface of the antigen it overflows through the side-arm and into an Erlenmeyer flask only to be reheated and recondensed to continue the process. The condenser and side-arm tube are cooled continuously with running tap water. In the tables and figures which follow, these extracted antigens are tested with precipitating antisera made to extracted antigens; only native antigens are tested with antisera made to native antigens.

The total and non-protein nitrogen of all native and extracted antigens were determined by the Folin-Wright method (1919), a modified Kjeldahl, and the protein nitrogen calculated and converted to grams of protein per hundred milliliters of serum. These values are shown in Table I.

All antisera were made in a similar manner, i.e., four injections of doubling doses of antigen on alternate days, the initial dose being 5 mgs. per kg. of body weight of rabbit. The rabbits were bled 14 days after the last injection.

The biochemical similarity of the antigens was determined by the precipitin ring test as described by Boyden and Noble (1933), in which .5 ml. of doubling dilutions of buffered (pH 7.0) antigen and .1 ml. of

antiserum are incubated at 37.5° C. for one hour. These results were then compared with those obtained by a new method employing the Libby (1938) photronreflectometer, a photoelectric nephelometer.

This instrument provides a constant source of parallel rays of light from an electric bulb which are permitted to pass through the turbid systems produced by the interaction of antigens and antibodies. Light rays reflected from the suspended particles fall upon a photoelectric cell and generate a current of electricity which causes a deflection on a galvanometer. In this manner the instrument was used to indicate the relative turbidities formed at 37.5° C. when 1.2 ml. of doubling dilutions of buffered (pH 7.0) antigen and .2 ml. of antiserum were incubated for 20 minutes. In all tests dilutions are in terms of protein rather than parts of serum. The galvanometric readings furnish the

TABLE I

Serum antigens used in experiments

Scientific name	Common name	Grams protein per 100 ml.
Gallus domesticus	leghorn	3.90
Gallus domesticus	plymouth rock	3.55
Gallus domesticus	buff orpington	4.20
Numida meleagris	guinea hen	2.82
Phasianus c. colchicus	pheasant	2.80
Meleagris gallopavo	turkey	3.95
Anser domesticus	domestic goose	3.94
Anas domesticus	domestic duck	3.50
Cath. a. septentrionalis	turkey buzzard	2.94
Pelecanus erythrorhynchos	pelican	3.20
Larus californicus	western gull	3.76
Drom. n. novae-hollandiae	emu	2.30

points for a curve, the area under which is used to determine the reaction of an antigen with an antiserum over a wide range of antigen dilutions. Comparison of the areas under the curves constructed from homologous and heterologous reactions furnishes evidence of the degree of chemical similarity between any two antigens.

The ring test, consisting of a series of tubes with doubling dilutions of antigen, is a very delicate and sensitive test. The slightest jarring or convection currents of the water bath may disturb the fine ring present in the titer tube, giving a false reading. All ring tests were run at least three times, many were tested six or more times. In only a few cases did a series vary by more than one tube on repetition. Considering that a variation of one tube, because of the doubling dilutions, represents a large error, the need for at least three readings is obvious. With the photronreflectometer, the variability of repeated determinations of the total areas was found to be within three per cent.

Results

The following tables indicate some of the results obtained when the native and extracted antigens were tested with precipitating antisera by ring and photronreflectometric tests. The homologous titer indicated over each antiserum is in terms of protein rather than nitrogen content.

In the body of the table the homologous titers and areas are valued at 100 per cent. The heterologous titers and areas are expressed in per cent values which indicate their relation to the homologous values. A dash indicates that no test was made.

Figure 1 is a graphic representation of the total area of reaction between a diluted (1 + 2) (one part serum plus two parts of buffered saline) anti-leghorn serum and each of several antigens. While the even-numbered tube numbers have been omitted from the figure to prevent over-crowding, the turbidity values obtained with the even-numbered tubes will be found on the graph. In all figures, tube number 1 is a dilution of one part of protein to 31.75 parts of saline; tube number 2 is a dilution of one part of protein to 62.5 of saline; tube number 3 a dilution of one part to 125; each succeeding tube possessing one-half of the concentration of protein of its predecessor.

In a similar manner the crystalline egg albumins, hemoglobins, and lens-proteins were used to determine relationships. Six antisera made to the hemoglobins of leghorn, duck, and turkey could not distinguish between the heterologous and homologous antigens by ring test. A similar condition was experienced with five antisera made to the lens proteins of leghorn, duck, and turkey. With the photronreflectometer, however, the same antigens and antisera reacted to give values comparable to those obtained with the serum-proteins. The following table shows some of the values indicated by ring and photronreflectometric tests.

Discussion

The results tabulated here represent a portion of a problem which has been in progress for a period of over four years. During this time a total of over 4,000 ring tests and several hundred turbidity curves have been completed. It is from this larger number of tests, and not only from the typical ones illustrated, that materials for the discussion and conclusion have been obtained.

Just as in a typical taxonomic study based on morphological data, similarities and differences of varying degrees of conservatism are revealed, so serological studies indicate the presence of substances

common to all of the birds but possessing sufficient differences to permit their differentiation. The fact that the sera of members of the various orders of birds reacted with most of the precipitating antisera is a

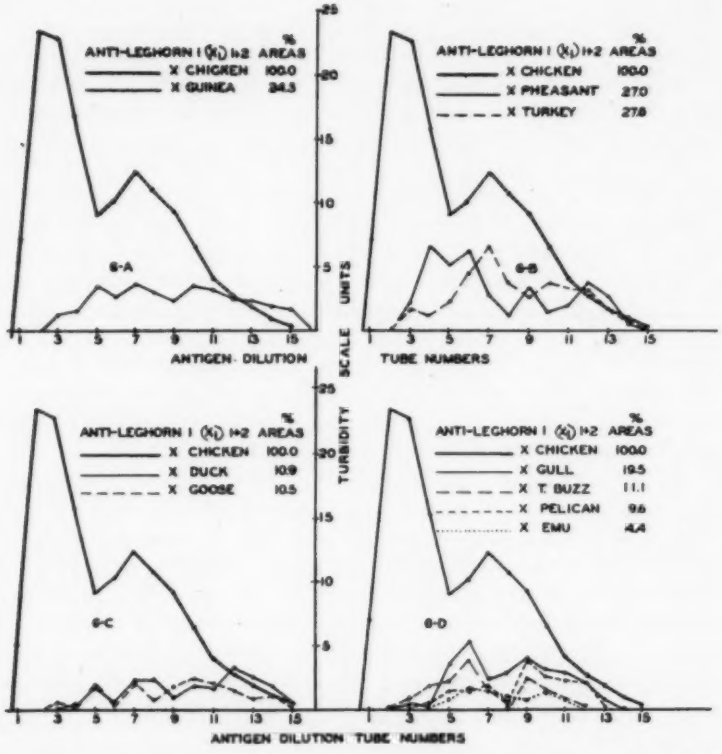

FIG. 1. Graphic representation of areas described by the interaction of a single antiserum (leghorn) with each of several antigens. The turbidity readings for the varying dilutions are plotted along the abscissa. Tube number 1 is a dilution of one part of protein to 31.75 parts of saline; tube number 2 is a dilution of one part of protein to 62.5 of saline; tube number 3 a dilution of one part to 125; each succeeding tube possessing one-half of the concentration of protein of its predecessor. Note the bimodality, or polymodality, of these curves which is typical of reactions in which sera serve as antigens. (Reprinted by permission from DeFalco, 1941, *Proc. Soc. Exper. Biol. and Med.*, **46**: 500.)

strong indication of the chemical similarity of all the birds, especially since the antisera were produced to a single series of injections. Similar single-series all inclusive precipitating antisera for other classes of

vertebrates are rare, if at all possible. Unpublished data of Dr. D. G. Gemeroy of this department reveal the difficulties encountered in demonstrating a link between "closely related" fishes even with very strong precipitating antisera. Results obtained with multiple series antisera made to amphibian sera by Boyden and Noble (1933) and to mammalian sera by Wolfe (1939) have indicated the limited range of reactivity of these antisera. This is most probably due to the greater degree of evolutionary divergence in the chemical constituents of these sera. Thus it seems clear that birds are the most homogeneous class of vertebrates both morphologically and serologically. The tables and figures indicate, however, that chemical differences among birds may be readily demonstrated by the precipitin reaction.

With some exceptions there is a general agreement between the relative systematic order indicated by the more objective serological techniques and the relative systematic order obtained by the more subjective morphological comparisons of the taxonomist. One very striking exception in this series of tests is the relationship of the guinea hen to the other domestic chickens. It may be noted in Table II that the relationship of the guinea hen to leghorn is 1.6 per cent. Approximately the same value was obtained with four other antisera. In a study with the lens and hemoglobins of these animals, however, a much greater degree of relationship was indicated. An attempt to ascertain the cause of the discrepancy between these two sets of data revealed that the ratios of serum proteins (basis for ring test) were quite different.

Leghorn	0.74 (albumin) to 1.0 (globulin)
Guinea hen	3.60 (albumin) to 1.0 (globulin)

Thus at comparable dilutions the guinea hen serum possesses about one-fifth as much globulin as the leghorn. Add to this the fact that the guinea hen globulin is different qualitatively and we may have the cause for the unexpected low value of 1.6 per cent. With the reciprocal test the fact that more but yet different globulin is present in the leghorn serum may account for the higher value of 25 per cent. Studies in this laboratory have shown that the globulin fraction is usually a better stimulant of antibody production than is albumin. If this is true, then the serological relationship between guinea hen and leghorn should be based on comparable amounts of active antigen rather than comparable amounts of total protein. A more correct basis for comparison would, therefore, be provided by tests of antisera made to purified fractions and tested with equivalent amounts of the same kind of protein in all the species compared.

TABLE II

Sera (native and extracted)	Titer 512,000 Antileghorn serum (to native) × native sera		Titer 2,048,000 Antileghorn serum (to extracted) × extracted sera		Titer 128,000 Antiturkey serum (to native) × native sera		Titer 2,048,000 Antiturkey serum (to extracted) × extracted sera		Titer 1,024,000 Antiduck serum (to native) × native sera		Titer 2,048,000 Antiduck serum (to extracted) × extracted sera	
	RN*	PN*	RE*	PE*	RN*	PN*	RE*	PE*	RN*	PN*	RE*	PE*
Leghorn	100.0	100.0	100.0	100.0	25.0	53.2	50.0	—	0.8	—	6.3	7.8
Plymouth	100.0	102.1	100.0	—	25.0	53.0	25.0	48.8	0.8	28.7	3.1	—
Buff Orpington	100.0	97.9	100.0	—	25.0	—	50.0	—	0.8	—	6.3	—
Guinea Hen	1.6	24.5	1.6	7.0	25.0	18.2	25.0	—	0.8	—	0.8	—
Pheasant	50.0	27.0	25.0	41.8	12.5	37.8	50.0	—	0.4	—	1.6	—
Turkey	50.0	27.6	50.0	63.7	100.0	100.0	100.0	100.0	0.8	20.7	6.3	—
Goose	3.1	10.5	1.6	—	6.3	7.2	25.0	4.4	50.0	70.0	50.0	37.1
Duck	3.1	10.9	1.6	5.5	3.1	—	25.0	3.9	100.0	100.0	100.0	100.0
Turkey Buzzard	3.1	11.1	0.8	—	0.4	7.8	12.5	—	6.3	—	1.6	—
Pelican	3.1	9.6	3.1	—	0.0	13.6	6.3	—	1.6	10.3	3.1	1.7
Gull	0.0	19.5	0.8	11.1	1.6	—	12.5	—	0.4	—	0.8	—
Emu	0.4	4.4	0.8	—	0.0	—	6.3	—	0.4	—	0.8	—

* RN = Ring test values obtained using native sera against an antiserum made to native leghorn sera.
PN = Photronreflectometer values using same sera and antiserum as in RN.
RE = Ring test values obtained using extracted sera against anti-extracted leghorn sera.
PE = Photronreflectometer values using same sera and antiserum as in RE.

In the body of Table II may be noted the ring test value of 0.0 per cent, for gull serum when tested with an anti-leghorn sera failed to produce rings yet gave a value of 19.5 per cent when tested with one of these antisera by the photronreflectometric method. At present this cannot be explained.

The bimodality exhibited by the interaction of complex antigens, such as sera, with antisera (as illustrated by Figure 1) is typical of these antigens. Simple antigens, such as hemoglobins, lens-proteins and crystalline egg albumins have quite consistently exhibited unimodal curves indicating the probability of the presence of a single anti-

TABLE III

Sera (native and extracted)	Titer 2,048,000 Antiguinea serum (to native) × native sera		Titer 1,024,000 Antiguinea serum (to extracted) × extracted sera		Titer 1,024,000 Antipelican serum × native sera	
	RN*	PN*	RE*	PE*	RN*	PN*
Leghorn	25.0	32.5	25.0	—	0.8	30.9
Plymouth	25.0	—	25.0	—	0.8	—
Buff Orpington	25.0	—	25.0	—	0.8	—
Guinea Hen	100.0	100.0	100.0	—	1.6	19.7
Pheasant	50.0	34.7	25.0	—	0.4	—
Turkey	25.0	22.0	25.0	—	0.8	—
Goose	25.0	9.8	6.3	—	0.8	—
Duck	12.5	6.8	3.1	—	3.1	3.7
Turkey Buzzard	12.5	5.1	0.0	—	6.3	18.3
Pelican	12.5	2.4	3.1	—	100.0	100.0
Gull	25.0	5.8	0.8	—	1.6	26.5
Emu	12.5	0.1	0.0	—	1.6	—

* RN = Ring test values obtained using native sera against an antiserum made to native leghorn sera.
PN = Photronreflectometer values using same sera and antiserum as in RN.
RE = Ring test values obtained using extracted sera against anti-extracted leghorn sera.
PE = Photronreflectometer values using same sera and antiserum as in RE.

genic substance. It may be of particular interest to note that, contrary to popular belief, lens-proteins may be readily distinguished. This separation, however, is not easily afforded by the ring test but is accomplished with comparative ease with the photronreflectometer.

The injection of a mixture of antigens, such as serum, may result in the production of: (a) antibodies to one fraction alone; (b) antibodies to several or all fractions, with more to one fraction than to another. This may be easily demonstrated by testing several antisera made to a mixed antigen with its purified constituent parts. In sera, the various protein fractions are rarely present in equal amounts, so the ring test

endpoints may, in some cases, be the result of one fraction and its antibodies, and in other cases the result of another fraction with its antibodies. Since in relationship studies the antiserum is kept constant, a small but strongly antigenic fraction spends the greater part of its reaction within the first few tubes, whereas a larger, but less antigenic, fraction yields a wider prozone and positive reactions occur in the higher dilutions of antigen.

In view of the variability in the response of the rabbit (the usual antibody-producer), and because three antisera made in exactly the same manner may differ greatly in specificity, it is very unlikely that

TABLE IV

Source of Antigen	Titer 2,048,000 Anti-leghorn Crystalline Egg Albumins Serum		Titer 2,048,000 Anti-turkey Hb Serum		Titer 1,024,000 Anti-turkey lens Serum	
	RN*	PN*	RN*	PN*	RE*	PE*
Leghorn	100.0	100.0	100.0	52.0	100.0	80.4
Guinea Hen	—	—	100.0	30.9	—	40.5
Pheasant	—	—	100.0	74.8	—	—
Turkey	100.0	34.0	100.0	100.0	100.0	100.0
Goose	50.0	16.0	100.0	—	100.0	11.8
Duck	50.0	26.4	100.0	—	100.0	11.4
Turkey Buzzard	—	—	100.0	34.0	—	—

* RN = Ring test values obtained using native sera against an antiserum made to native leghorn sera.
PN = Photronreflectometer values using same sera and antiserum as in RN.
RE = Ring test values obtained using extracted sera against anti-extracted leghorn sera.
PE = Photronreflectometer values using same sera and antiserum as in RE.

reciprocal tests would often confirm the original tests. In those cases in which antigens are ring-tested with pooled antisera the internal compensation of the mixture might conceivably make for better agreement between original and reciprocal tests. Possibly because of the more adequate data obtained by photronreflectometric methods, in contrast to the ring-test, the reciprocal tests show better agreement with the original tests than is seen by ring tests.

There is considerable evidence to indicate that the antibodies responsible for the sensitivity, or titer, of an antiserum may not be the same antibodies responsible for the greater part of the precipitating capacity of that antiserum. Several antisera, made in the course of this study, possessed titers of 128,000 or more, and yet, photronreflectometrically the turbidity at various dilutions of antigen was zero.

Other antisera with a titer of 10,000,000 or more produced less precipitate than others with a titer of 512,000. Wolfe and Baier (1938) have had a similar experience as indicated by the statement in their summary, "Indications are that at least two types of antibodies are present: (1) a 'titer'-producing antibody and (2) a 'precipitate'-forming antibody."

While the correlation between the precipitating capacities and the sensitivities of antisera appears to be weak and irregular, there is, nevertheless, an almost perfect parallelism in the systematic position of the birds as revealed by relationship values derived from ring and turbidity tests.

It would be difficult at this time to state with any degree of certainty what the effect of petroleum-ether extraction was upon the antigens. The differences between relationship values obtained by use of unextracted (native) materials and those obtained with extracted antigens are of the same magnitude as might be expected by injecting two rabbits with the same material; the rabbit being the greatest variable. In experiments with antisera made to extracted sera it has been noted repeatedly that while the sensitivity of these antisera is just as high as those made to the unextracted sera, the precipitating capacity of the former is in all cases decidedly smaller than the latter. This is not true of extracted lens proteins and extracted crystalline egg albumins.

Table V shows the ring test values obtained when both native and extracted antigens are tested with antisera made to each of them. Antisera made to native antigens react more strongly with native than with extracted antigens. Also, antisera made to extracted antigens react more strongly with extracted antigens than do native antigens. This would imply that extraction with petroleum-ether leaves behind a protein molecule whose surface configuration is somewhat different from that before extraction. Apparently, the portion of the molecule least affected is that responsible for the antigenic specificity, for the greater losses are found with the heterologous reactions. In some cases (such as plymouth rock $\times$ anti-native guinea $hen_{\times 2}$) there must have been considerable denaturation of the antigen to give such a low value. This could not be detected, however, as precipitated material or by nitrogen determination which accounted for all the protein.

The fact that almost all photronreflectometric curves were unimodal when extracted antigens were used, and that all anti-extracted sera were poorer precipitating sera, might well indicate the inactivation of a portion of the antigen. Fortunately, it appears that extraction is not necessary in avian studies. Should it become a necessary procedure

in the study of closely related forms, attempts should be made with extracting agents less destructive to protein than petroleum ether, possibly sodium ricinoleate.

The close relationship of gallinaceous birds as determined by morphological methods is corroborated by both ring and photronreflectometric tests. The close relationship of the Anseres (duck and goose) is likewise confirmed. The distant relationship of the flightless birds (primitive), represented by the emu, to the gallinaceous birds, is con-

TABLE V

The effect of extracting antigens with petroleum-ether on relationship values

Antigens	Anti-native guinea hen sera		Anti-native leghorn sera		Anti-extracted leghorn serum	Anti-extracted turkey serum
	×1	×2	×1	×2	×1	×2
Native guinea	100.0	100.0	1.6	1.6	0.0	12.5
Extracted guinea	50.0	100.0	0.8	0.8	1.6	25.0
Native plymouth	25.0	50.0	100.0	100.0	12.5	50.0
Extracted plymouth	12.5	0.4	100.0	100.0	100.0	50.0
Native buff orp.	25.0	50.0	100.0	100.0	12.5	50.0
Extracted buff orp.	25.0	12.5	100.0	50.0	100.0	50.0
Native turkey	12.5	25.0	25.0	50.0	12.5	100.0
Extracted turkey	3.1	1.6	6.3	25.0	50.0	100.0
Native duck	12.5	12.5	6.3	1.6	0.0	6.3
Extracted duck	0.8	0.8	0.0	0.4	1.6	25.0
Native gull	25.0	3.1	0.0	0.0	0.0	3.1
Extracted gull	3.1	0.0	0.0	0.0	0.8	12.5

firmed by these tests. That the birds as a class constitute a homogeneous group chemically is clearly indicated throughout these tests.

SUMMARY AND CONCLUSIONS

1. Chemical resemblances and differences between avian sera are demonstrated by precipitating antisera.

2. Two techniques are employed to determine the interaction between antigen and antibody. The ring test, though revealing only the end point, has the advantage of greater sensitivity. The photronreflectometer measures the degree of reaction between antigen and antibody over the entire range of dilutions within which a demonstrable reaction occurs.

3. Tests with antisera made to extracted sera show that extraction of the antigens causes the production of poorer precipitating antisera, that is, few antibodies, or weaker ones.

4. All antisera non-specific by ring test were specific by photron-reflectometric test.

5. The systematic position of the birds is generally confirmed by these tests.

6. Birds are serologically an essentially homogeneous group.

7. Extraction of antigens with petroleum-ether is not necessary in avian serological studies. If found necessary in studies with closely related forms it is suggested that a less destructive method, or reagent, or both, be found.

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LITERATURE CITED

BAIER, JOSEPH G., JR., 1933. Quantitative studies on precipitins. *Physiol. Zool.*, **6**: 91–125.

BAIER, JOSEPH G., JR. AND HAROLD R. WOLFE, 1942. Quantitative serologic relationships within the Artiodactyla. *Zoologica*, **27**: 17–23.

BOYDEN, ALAN, AND JOSEPH G. BAIER, JR., 1929. A rapid quantitative precipitin technique. *Jour. Immun.*, **17**: 29–37.

BOYDEN, ALAN, AND G. KINGSLEY NOBLE, 1933. The relationships of some common Amphibia as determined by serological study. *Am. Mus. Novitates*, No. **606**: 1–24.

BOYDEN, ALAN, 1939. Serological study of the relationships of some common invertebrata. *Yearbook, Carnegie Institution of Washington*, **38**: 219–220.

BOYDEN, ALAN, 1942. Systematic serology: a critical appreciation. *Physiol. Zool.*, **15**: 109–145.

COHEN, BERNICE, 1939. Unpublished thesis incorporated in a paper by H. R. Wolfe, Standardization of the precipitin technique and its application to studies of relationships in mammals, birds and reptiles. *Biol. Bull.*, **76**: 108–120.

CUMLEY, R. W., AND M. R. IRWIN, 1940. Differentiation of sera of two species of doves and their hybrid. *Proc. Soc. Exp. Biol. and Med.*, **44**: 353–355.

DEFALCO, RALPH J., 1941. A comparison of antigens by interfacial and nephelometric methods. *Proc. Soc. Exp. Biol. and Med.*, **46**: 500–502.

ERHARDT, ALBERT, 1930. Die Serodiagnostichen Untersuchungen in der Ornithologie. *Jour. fur Ornithologie*, **78**: 214–234.

FOLIN, OTTO, AND L. E. WRIGHT, 1919. A simplified macro-Kjeldahl method for urine *Jour. Biol. Chem.*, **38**: 461–464.

GRAHAM-SMITH, G. S., 1904. Blood relationship amongst the lower vertebrata and arthropods, etc. as indicated by 2,500 tests with precipitating antisera. In Nuttall, Blood Immunity and Blood Relationship. Section VIII, Cambridge, 1904.

HOPKINS, F. GOWLAND, AND S. N. PINKUS, 1898. Observations on the crystallization of animal proteids. *Jour. Physiol.*, **23**: 130–136.

LIBBY, RAYMOND L., 1938. The photronreflectometer—an instrument for the measurement of turbid systems. *Jour. Immun.*, **34**: 71–73.

SASAKI, KIYOTSUNA, 1928. Serological examination of the blood-relationship between wild and domestic ducks. *Jour. of the Dept. of Agriculture, Kyushu Imperial University*, **2**: 117–132.

WELKER, W. H. AND C. S. WILLIAMSON, 1920. Hemoglobins. I. Optical constants. *Jour. Biol. Chem.*, **41**: 75–79.

WILHELMI, RAYMOND W., 1942. The application of the precipitin technique theories concerning the origin of vertebrates. *Biol. Bull.*, **82**: 179–189.

WOLFE, HAROLD R., AND JOSEPH G. BAIER, JR., 1938. Comparison of quantitative percipitin techniques as influenced by injection procedures. *Physiol. Zool.*, **11**: 63–74.

THE RESISTANCE AND ACCLIMATIZATION OF MARINE FISHES TO TEMPERATURE CHANGES. I. EXPERIMENTS WITH GIRELLA NIGRICANS (AYRES) [1]

PETER DOUDOROFF

(Scripps Institution of Oceanography, University of California, La Jolla [2])

Introduction

Fishes occur in nature over a wide range of temperature, from the freezing point of sea water (about −2°) to at least 37° C. (Sumner and Sargent, 1940) or higher. The range of tolerance of most species apparently does not include both of these extremes. Differences of resistance to extreme temperatures shown by different species, both under experimental conditions (Huntsman and Sparks, 1924; Huntsman, 1926; Hathaway, 1927) and in nature (Storey, 1937), have been found to correspond to differences of distribution (geographic or vertical) in relation to temperature.

Quantitative studies of acclimatization, as a factor in the resistance of fishes to high temperatures, have been undertaken repeatedly (Loeb and Wasteneys, 1912; Hathaway, 1927; Binet and Morin, 1934; Sumner and Doudoroff, 1938). Detailed studies of acclimatization to low temperatures are lacking. This oversight is not surprising. The view that low temperatures are less injurious than equally extreme high temperatures, at least when freezing is not involved, is not uncommon. Heretofore, Maurel and Lagriffe (1899a, b, c) have attempted to compare experimentally the upper and lower limits of tolerance of some, chiefly fresh-water, fishes. They concluded, "Qu'ils semblent mieux organisés pour résister aux températures extrêmes du froid qu'aux températures extrêmes de la chaleur." It is not clear what criterion was used in judging whether a given temperature was more or less "extreme" than another. Aside from the fact that the methods used were not entirely satisfactory, it appears that the above conclusion was probably based upon some faulty line of reasoning, and is not an obvious outcome of the experimental results.

In general, the sensitivity of fishes to chilling has received little attention from experimental physiologists, who have most frequently

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[2] Now with the Louisiana State Department of Conservation, University, Louisiana.

used relatively cold-hardy fresh-water and estuarine marine fishes as laboratory material. The survival of fishes which had been frozen for a long time in water, or frozen solid throughout in air, has been reported by a number of early observers (for references see Borodin, 1934). This has not been confirmed by the more reliable and recent investigations, but it has been shown that some cold-hardy fishes can survive super-cooling (i.e., without freezing) to temperatures below the freezing point of their body fluids, and also brief and partial freezing of their superficial tissues (Regnard, 1895; Britton, 1924; Weigmann, 1930, 1936; Borodin, 1934; Schmidt, Platonov and Person, 1936; Luyet, 1938). The phenomenon or possibility of death after more prolonged exposure to moderately low (above-zero) temperatures has attracted much less attention. Indeed, Luyet and Gehenio (1938), in the "compendium" of their extensive review dealing with the lower limit of vital temperatures, state: "The third group, namely that of organisms which are killed at above-zero temperatures, includes only the homoiotherms and some of the higher plants." A number of observations on the death of poikilotherms from chilling are, however, on record (e.g., Weigmann, 1929).

Changes of cold-tolerance, resulting from continued exposure to high and low temperatures, have been observed in fishes by Wells (1935) and Sumner and Doudoroff (1938), but accurate quantitative studies of their magnitude were not undertaken. There is also surprisingly little evidence available for an influence of acclimatization upon susceptibility to chilling in other animals.

The term acclimatization will be used here to designate only gradual changes in the effect upon an organism of a given environmental factor, which can be produced experimentally in the individual organism by altering its environment with respect to this particular factor. Heilbrunn (1937) introduces his interesting chapter on acclimatization with a fairly comprehensive discussion of the modern meaning of the term. However, Heilbrunn cites as examples of temperature acclimatization certain seemingly adaptive differences between individuals of the same species from different latitudes, as well as seasonal variations of resistance to extreme temperatures, etc. (e.g., Hörstadius, 1925; Bodenheimer and Klein, 1930). This is not entirely consistent with his own definition. Such variations may be inherent, i.e., sub-specific (resulting from mutation and selection) or inherently periodic, or they may be related to differences of diet or to environmental differences other than those of temperature. The use of the more general term "adaptation" (Bělehrádek, 1935; Fox, 1939) is

preferable in referring to such variations, which have not been shown to be due to acclimatization alone, as defined here.

True acclimatization to cold, as indicated by changes of resistance to freezing (sub-zero temperatures), has been studied in insects by Payne (1926a, b) and by Mellanby (1939). Mellanby (1939, 1940) also investigated the influence of acclimatization upon resistance to immobilization by cold at non-freezing temperatures (chill-coma) in insects and amphibia. The influence upon survival at such temperatures was not studied in detail. Ogle and Mills (1933) produced changes of cold-tolerance in rabbits. The mechanisms of acclimatization in cases in which death is associated with a thermoregulative breakdown (in strict homoiotherms), or with the freezing of body fluids, are probably quite different from that involved in changes of resistance of poikilotherms to chilling.

The acclimatization of animals to heat is well known. Studies with fishes have already been cited. Heilbrunn (1937) cites other references.

The present paper deals with quantitative experiments on the resistance of the marine fish *Girella nigricans* to extreme temperatures and on the phenomena of acclimatization to heat and especially to cold, and with their ecological significance. Experiments with other species and a discussion of some possible causes of death at extreme low temperatures will be presented in a later publication.

I am indebted to Dr. F. B. Sumner, of the Scripps Institution of Oceanography, for his invaluable advice and unfailing interest during the progress of this investigation. Grateful acknowledgment is also given to other members of the staff of the Scripps Institution for helpful suggestions, information and assistance.

Material

The young (immature) greenfish, *Girella nigricans* (Ayres), used in the experiments were taken in the intertidal area near the Scripps Institution, La Jolla, California. This marine shore-fish is an active and fairly hardy inhabitant of the littoral zone, occurring in open water and in tide-pools along the open coast.

For proper evaluation of the results, with respect to their ecological implications, information relative to temperature conditions to which the animals are normally exposed in their natural habitat is necessary. The following data are based on daily records of water temperatures at the end of the Scripps Institution's 1000-foot pier. The average surface temperature for 23 years (1916 to 1938) was 17.0° C. The average for January and February, the coldest months, was 14.0°, and that for July and August, the warmest months, was 20.6°. The lowest

and highest monthly averages on record were 11.8° and 23.6°, respectively. Corresponding weekly averages were 11.7° and 25.1°. More significant as indices of the normal temperature range are the mean yearly minimum and maximum, that is, means of the lowest and highest temperatures, respectively, occurring during each summer and each winter. Since the daily records were not available to the writer, means of the lowest and highest weekly averages were calculated. These values were 13.3° and 22.0°. The average bottom temperature at a depth of 5 meters was 0.8° lower than at the surface. This vertical gradient averaged 1.9° in 5 meters during the two warmest months. The range of daily temperatures at a given level within a single week sometimes exceeds 5° or even 7°.

In tide-pools in which some Girella remain during low tide the temperature generally does not differ greatly from that in open water, but occasionally temperatures above 26° have been observed. According to Jordan, Evermann and Clark (1930), the range of distribution of *G. nigricans* extends along the "coast of California from Monterey to Cape San Lucas," i.e., further southward than northward from La Jolla.[3] This probably does not represent the extent of the normal habitat of the species. Since different physiological races or subspecies may be involved, experimental results obtained with specimens from one locality are not strictly applicable to those from other localities.

Methods

In comparing the resistance of fishes to extreme temperatures, some workers (e.g., Loeb and Wasteneys, 1912) determined the average duration of survival at constant lethal temperatures. Others (Maurel and Lagriffe, 1899a, b; Huntsman and Sparks, 1924; Sumner and Doudoroff, 1938; et al.) determined the temperature at which death or coma occurred when the water was warmed or cooled fairly rapidly. These methods are satisfactory for some purposes, but the results are not readily applicable to many ecological problems. The ecologist is interested in knowing and comparing the most extreme temperatures at which organisms can survive indefinitely, or at least for equal and long periods of exposure. That the limit of tolerance varies with the duration of exposure involved is well known, and one may not assume that closely parallel relations always hold between temperatures just tolerated for brief periods and ones tolerated for long periods, especially by different species.

More suitable for present purposes was the method used by Hathaway (1927) in experiments on heat-tolerance. It involved the use of

[3] For data on surface temperatures in this range see U. S. Weather Bureau (1938)

a series of constant test temperatures and fixed periods of exposure. Hathaway's calculated "average tolerance limits," do not, however, actually represent what the term implies, being lower than the true values. For any given period of exposure he regarded a given temperature as being the limit of tolerance for a percentage of the experimental animals obtained by taking the difference between the percentages surviving at this temperature and at the next higher (more extreme) test temperature. The formula used yielded the mean of the "tolerance limits" thus obtained. Yet the interval between the two test temperatures corresponds to a statistical "class-interval," into which fall the individual tolerance limits of the above percentage of specimens. The usual statistical procedure is to assume uniform distribution over the class-interval, and in computing the mean, the mid-value (not the lower limit) of each class is taken as the average value for all items in that class.

In the present experiments, instead of the mean, the *median tolerance limit* was adopted as a measure of resistance. The upper or lower median tolerance limit (T_m) for any given period of exposure will be defined as a temperature at which just 50 per cent of the specimens under consideration are able to survive for this period of time.[4] This value is more easily estimated than the mean tolerance limit, and is not affected by occasional extreme variants, such as unhealthy individuals.

The fishes were held in large tanks supplied with running sea water. Constant temperatures were maintained by thermostatic control or otherwise, and did not deviate from the stated values by more than 0.5° C. (usually less). When temperatures above 20° were maintained. the removal of excess air (supersaturation) was provided for by bubbling a stream of compressed air through the heated water before it entered the aquaria.

In testing their heat-tolerance, the fishes were transferred to an aquarium supplied with an ample flow (about one liter per minute) of heated and air-equilibrated water. The temperature was kept constant by thermostatic control and generally did not deviate from the stated values by more than 0.1°, the maximal deviation allowed being 0.2°. The same degree of accuracy was maintained in cold-tolerance tests. These were performed in two ways. In early (long-time) experiments the aquarium was supplied with cooled (but not aerated) running water. In later experiments, in which the periods of exposure were limited to 24 hours, the tests were made in about nine liters of standing water in glass battery jars immersed in a constant-

[4] Obviously, equal numbers of specimens have tolerance limits above and below this value; hence the designation "median."

temperature tank. The water in this case was well aerated with compressed air. In a given series of experiments the same method was used throughout.

The determination of the upper or lower median tolerance limit of a group of fishes for any desired period of exposure was made in the following manner. A number of specimens (usually ten or more) were transferred directly to the test tank, maintained at a temperature which was believed to be close to the limit of tolerance. At the end of the desired time interval the number of specimens surviving was noted. If exactly 50 per cent of those tested survived, this temperature was taken as the median tolerance limit. If more or less than 50 per cent survived, similar tests were performed at other temperatures 1° C. (or, in a few instances 0.5°) apart, until two temperatures were found at one of which more than half and at the other less than half of the specimens tested survived. The median tolerance limit was then estimated by graphical interpolation. The percentage surviving at each of the two temperatures was plotted against the temperature on arithmetic co-ordinate paper, and the two points connected by a straight line. The temperature which corresponded to 50 per cent survival on this graph was taken as the median tolerance limit.[5] What is meant by survival will be explained later.

The maximal error in the estimation of the median which may result from the assumption of a straight-line relationship between the experimental points is, for present purposes, negligible, not exceeding a few tenths of one degree. The differences of tolerance in which the writer was interested were so clear-cut that the computation of the probability of their significance would be superfluous.

Results

Effects of Extreme Temperatures and the Criterion of Survival Adopted

At rapidly lethal high temperatures great stimulation, loss of equilibrium, and finally permanent and almost simultaneous cessation of respiration and of other movements (heat-coma) were observed. At slowly lethal high temperatures stimulation, disturbances of equilibrium and other signs of distress (initial shock effects) were frequently observed directly after transfer from low temperatures. This was followed by temporary recovery and more or less normal behavior. Specimens conditioned to high temperatures began to show distress only a few hours or minutes before death. Thus, neither recovery from the initial effects, nor failure to show any distress after transfer to a

[5] The upper median tolerance limit is identical with the "thermal index" used by Whitney (1939) in comparing the heat-resistance of mayfly nymphs.

high temperature, is an indication that the fish will survive the change. Recovery from heat-coma after return to a normal temperature was never observed in Girella.

At lethal low temperatures the initial shock effect was generally more pronounced than at high temperatures. It ranged from mild distress and disturbance of equilibrium to a violent, convulsive paroxysm, followed by cessation of respiratory and all other movements. I shall refer to the latter state as "primary chill-coma." At very low temperatures the fishes died without showing any further visible signs of life, except, in some cases, a brief quivering of the opercles and fins. At less extreme temperatures the fishes recovered more or less from the initial shock. Regular respiratory movements, if they had ceased, were resumed, and frequently the fishes righted themselves and regained normal appearance and behavior. After some hours or days, however, they again showed increasing distress, and finally ceased to respire and to respond to stimulation. This stage, to which I shall refer as "secondary chill-coma," was soon followed by death.

The initial shock was not manifest until several seconds after transfer to the low temperature, and apparently was not due to stimulation of the cutaneous sense organs, but was produced only when the low temperature had penetrated internally, probably to the central nervous system. Accordingly, it was more delayed in large specimens than in small ones. By cooling the animals gradually the shock was sometimes apparently prevented or reduced without appreciably delaying the onset of secondary chill-coma.

If it occurred at all, spontaneous temporary recovery from primary chill-coma (i.e., resumption of respiratory and other movements, occurring at the low temperature at which the coma was produced) began within a few minutes or, at most, within half an hour. Thus, in Girella primary chill-coma was not a very pronounced phenomenon, except at very rapidly lethal temperatures. In most cases the initial shock effect, although intense, did not involve complete coma of appreciable duration. In other species, however, primary chill-coma, followed by temporary recovery, may be quite prolonged. If returned to a normal temperature within one hour, Girella in a state of primary chill-coma were frequently capable of rapid and permanent recovery. After the onset of secondary chill-coma, spontaneous recovery was never observed, but the fishes sometimes showed brief and partial or even complete recovery on warming, provided that they were returned to the normal temperature within a few minutes. The more slowly lethal the temperature and, accordingly, the more delayed the onset of

secondary coma, the less likely were the fishes to recover on warming. The administration of artificial respiration, by passing a stream of water through the mouth and over the gills, favored recovery.

The accurate quantitative comparison of relative tolerances requires the adoption of a uniform and convenient "criterion of survival," that is an end-point after which the organism is regarded as having succumbed to the effects of the lethal agent. It is best to adopt as an end-point the permanent (not spontaneously reversible) cessation of some important function which is among the first to be arrested. The time required for the attainment of an advanced stage of necrobiosis is not as accurately indicative of relative rates of injury, and may be greatly influenced by temperature and other factors independently of the rate of injury. The permanent cessation of respiratory and other movements, either spontaneous or induced by mechanical stimulation, presumably resulting from the functional inactivation of the central nervous system, apparently satisfies the above requirements.

In the determination of heat-tolerance the application of the end-point adopted (i.e., cessation of all spontaneous and reflex movements) presented no difficulty. The occurrence of two types of chill-coma introduced some difficulty. Primary chill-coma differs from chill-coma which has been described in insects (Mellanby, 1939) and other animals in that the fishes were capable of rapid adaptation to the low temperature, with consequent temporary recovery. In this respect it differs also from heat-coma. On the other hand, the onset of secondary chill-coma is comparable with that of heat-coma and a satisfactory end-point. Neither was ever followed by spontaneous recovery, and both apparently represented the onset of death, as the fishes failed to recover on return to normal temperatures or lost the ability to do so within a very short time. At very extreme low temperatures, at which no spontaneous recovery from primary coma was noted, an entirely comparable end-point was lacking. In practice, Girella were regarded as having succumbed if they had failed to resume movements at the end of one hour after transfer. Thereafter, as after the onset of secondary coma, spontaneous recovery was apparently impossible, and recovery on warming was only rarely observed.

By way of summary, it can be stated that a specimen was regarded as having succumbed if no movements, either spontaneous or induced by mechanical stimulation, could be detected, but no determinations of lower tolerance limits were made for periods of exposure shorter than one hour. In this way any uncertainty as to whether movements had ceased permanently at the time of observation or had been suspended temporarily was eliminated.

Time-Temperature Relations of Heat and Cold Tolerance and the Influence of Acclimatization

The specimens used in the first series of quantitative experiments were collected in the fall of 1939, and held at 20° for three and a half months.[6] On March 14, 1940, they were divided into three lots, which were acclimatized thereafter to 12°, 20° and 28°, respectively, for nearly two months before tolerance determinations were begun. Evidence to be presented later indicates that the fishes were then completely acclimatized to the new temperatures, that is, their tolerance had reached a constant value and would not change appreciably with more prolonged acclimatization. Therefore, the results of subsequent tests may be regarded as comparable, in spite of unavoidable differences in the duration of acclimatization previous to testing.

Groups of eight to 14 specimens of similar history were tested in running water at a series of low or high temperatures, usually 1° C. apart. The number surviving was recorded at fixed intervals of time, namely, after 1, 3, 6, 12, 24, 48 and 72 hours of exposure to the test temperatures, and sometimes also after other periods.[7] The lengths of the specimens ranged from 6.0 cm. to 9.2 cm. The mean lengths in the different groups under comparison were approximately equal, although those of lots tested at high temperatures were slightly greater than those of lots tested at low temperatures. Since no obvious correlation was noted between the duration of survival and the size of the individual specimens, the smaller differences between the mean lengths of the test lots could be of no great consequence.

The experimental data are presented in Tables I and II. From these data the lower and upper median tolerance limits for the various periods of exposure after which observations were made were estimated by the method described earlier and plotted against the duration of exposure in Figures 1 and 2. The resulting curves will be referred to as the time-temperature curves of cold-tolerance and of heat-tolerance, respectively.

From Tables I and II it may be deduced that among specimens of similar history the variability of the individual tolerance limits (i.e., of the lowest and highest temperatures which individual specimens are

[6] The appearance in the stock-tank of a fatal, seemingly infectious disease (dermatitis), which was later brought completely under control, necessitated this delay.

[7] Signs of external disease frequently appeared after prolonged exposure to extreme low temperatures, especially in specimens which had been conditioned to the higher acclimatization temperatures. Since it was desirable to avoid disease as a complicating lethal factor, and to deal only with the direct lethal effects of cold, determinations of lower limits of tolerance for periods longer than three days were not attempted with such specimens.

just able to withstand) for any given period of exposure is small. The difference between temperatures at which 100 per cent and 0 per cent survival was obtained averaged slightly over 2°, and in no case exceeded 3°. In contrast with this, marked differences of tolerance were shown by specimens conditioned to different temperatures. The lower median tolerance limits for the various fixed periods of exposure adopted (one to 72 hours) of fishes conditioned to 28° and to 20° differed, on the average, by 4.3°, and those of fishes conditioned to 20° and 12° by 4.0°. The corresponding average difference between the upper median tolerance limits of 20°- and 12°-conditioned specimens was 3.2°. There can be no doubt that these differences are significant. Only the heat-tolerance of specimens conditioned to 20° and to 28° did not differ appreciably.

The time-temperature curves of cold-tolerance (Figure 1) are dissimilar (not parallel). After 24 hours the lower median tolerance limits of 28°-conditioned specimens remained constant at 13°. This temperature may apparently be regarded as the "ultimate" or "true" lower median tolerance limit for these specimens, that is, the least extreme low temperature at which 50 per cent of the specimens are killed by cold, regardless of the duration of exposure. The ultimate limits of tolerance for specimens conditioned to 20° and to 12° cannot be stated, but can be judged by extrapolation to be slightly above 8° for the former, and somewhat above 5° for the latter.

It is noteworthy that, whereas for brief periods of exposure (one or three hours) the differences between the lower tolerance limits of fishes conditioned to 20° and 28° was considerably smaller than the difference between those of specimens conditioned to 20° and 12°, the reverse is true for longer periods of exposure (12 hours or more).[8] Short-time experiments obviously do not yield an entirely satisfactory measure of the changes of relative tolerance produced by acclimatization.

The time-temperature curves of heat-tolerance (Figure 2) tend to become parallel with the time axis sooner than the cold-tolerance curves. The ultimate or true upper median tolerance limits were apparently 31.4° for both the 28°-conditioned and 20°-conditioned specimens and 28.7° for the 12°-conditioned specimens. The absence of any difference between the 72-hour upper limits of tolerance of specimens conditioned to 28° and 20°, in contrast with a difference of

[8] The ratio (R) of the former difference to the latter $\left(R = \frac{T_m 28° - T_m 20°}{T_m 20° - T_m 12°}\right)$ increases with increasing time of exposure from 0.56 for one hour to 1.47 for 72 hours of exposure. When plotted against the logarithm of the duration of exposure to the test temperatures in hours ($\log t$), the values of R tend to fall roughly in a straight line ($R = 0.56 + 0.49 \log t$).

TABLE I

Per cent survival of Girella at various low temperatures and the estimated lower median tolerance limits (T_m) in relation to temperature of previous acclimatization and to time (duration) of exposure to the test temperatures

Date 1940	Acclim. temp. (° C.)	Test temp. (° C.)	No. of fish	Mean length (cm.)	Per cent surviving after							
					1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.
5/8	28°	13°	10	7.0	100%	100%	100%	100%	90%	50%	50%	
5/13	"	12°	11	7.1	100	100	91	45	0	0	0	
5/11	"	11°	10	7.1	100	100	90	30	0	0	0	
5/14	"	10°	10	7.2	100	100	0	0	0	0	0	
5/15	"	9°	10	7.2	100	90	0	0	0	0	0	
5/16	"	8°	10	7.2	80	10	0	0	0	0	0	
5/17	"	7°	10	7.0	20	0	0	0	0	0	0	
5/18	"	6°	8	7.1	0	0	0	0	0	0	0	
				T_m:	7.5°	8.5°	10.6°	12.1°	12.6°	13.0°	13.0°	
5/3	20°	8°	14	7.3	100%	100%	100%	100%	93%	64%	50%	
5/1	"	7°	12	7.2	100	100	100	58	17	0	0	
5/6	"	6°	14	7.3	100	93	36	0	0	0	0	
5/7	"	5.5°	12	7.2	75	42	0	0	0	0	0	
5/7	"	5°	14	7.3	0	0	0	0	0	0	0	
				T_m:	5.3°	5.6°	6.2°	6.9°	7.4°	7.8°	8.0°	
5/28	12°	5°	10	7.1	100%	100%	100%	100%	100%	100%	90%	50%
5/21	"	4°	11	7.1	100	100	100	100	100	45	0	0
5/25	"	3°	11	7.1	100	100	100	100	55	0	0	0
6/3	"	2°	10	7.2	90	70	60	10	0	0	0	0
6/4	"	1°	10	7.1	20	0	0	0	0	0	0	0
				T_m:	1.4°	1.7°	1.8°	2.4°	2.9°	4.1°	4.6°	5.0°

TABLE II

Per cent survival of Girella at various high temperatures and the estimated upper median tolerance limits (T_m) in relation to temperature of previous acclimatization and to time (duration) of exposure to the test temperatures.

Date 1940	Acclim. temp. (° C.)	Test temp. (° C.)	No. of fish	Mean length (cm.)	Per cent surviving after								
					1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.	Other periods
6/19	28°	33°	8	7.6	100%	100%	88%	0%	0%	0%	0%	0%	
6/21	"	32°	9	7.7	100	100	100	100	44	0	0	0	
6/24	"	31°	9	7.7	100	100	100	100	100	100	89	89	168 hrs.: 89%
			T_m:					32.5°	31.9°	31.5°	31.4°	31.4°	31.4° (168 hr.)
6/18	20°	35°	10	7.8	0%	0%	0%	0%	0%	0%	0%	0%	0.5 hr.: 50%
6/18	"	34°	12	7.9	100	0	0	0	0	0	0	0	
6/17	"	33°	12	7.8	100	100	25	0	0	0	0	0	
6/15	"	32°	12	7.7	100	100	100	75	0	0	0	0	
6/8	"	31°	12	7.8	100	100	100	100	100	83	83	83	
			T_m:		34.5°	33.5°	32.7°	32.3°	31.5°	31.4°	31.4°	31.4°	35.0° (0.5 hr.)
6/29	12°	31°	8	8.0	25%	0%	0%	0%	0%	0%	0%	0%	
6/28	"	30°	8	8.0	100	0	0	0	0	0	0	0	2 hrs.: 50%
6/22	"	29°	12	7.9	100	83	50	50	33	33	33	33	
6/19	"	28°	10	7.9	100	100	100	90	90	90	90	90	
6/14	"	27°	10	7.8	100	100	100	100	100	100	100	100	
			T_m:		30.7°	29.4°	29.0°	29.0°	28.7°	28.7°	28.7°	28.7°	30.0° (2 hr.)

5° between the corresponding lower limits, is of interest. A change of cold-tolerance produced by acclimatization is not necessarily accompanied by a corresponding change of heat-tolerance.

The lower the temperature of acclimatization, the earlier does the time-temperature curve of heat-tolerance tend to become parallel with

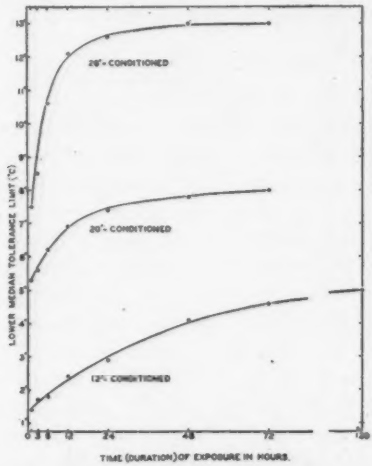

FIG. 1. Time-temperature curves of cold-tolerance: Lower median tolerance limits in relation to the time (duration) of exposure to the test temperatures. The specimens were previously acclimatized to three different temperatures (12°, 20° and 28°), indicated on each curve. Data from Table I.

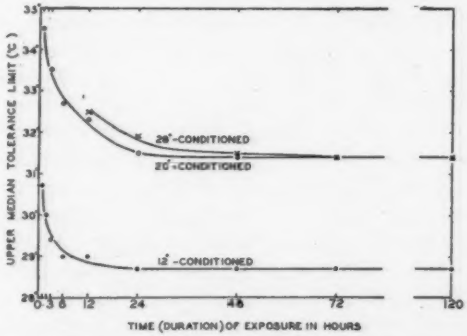

FIG. 2. Time-temperature curves of heat-tolerance: Upper median tolerance limits in relation to the time (duration) of exposure to the test temperatures. The specimens were previously acclimatized to three different temperatures (12°, 20° and 28°), indicated on each curve. Data from Table II.

the time axis. This is the reverse of the relationship between the corresponding curves of cold-tolerance (Figure 1). At slowly lethal temperatures the fishes probably undergo some acclimatization, the extent of which must vary with the magnitude of the change involved in the transfer to the lethal temperature. The differences noted above between the curves obtained with fishes acclimatized to different temperatures can be explained, at least in part, on the basis of this assumption.

The Rate of Acclimatization

Data already available relative to the rate of change of heat-tolerance of some marine fishes with acclimatization (Loeb and Wasteneys, 1912; Sumner and Doudoroff, 1938) indicate that increased heat-resistance is acquired rapidly after transfer to higher temperatures. While this change is reversible, heat resistance is apparently lost after transfer to lower temperatures much more slowly than it is gained. No detailed data were available on the corresponding rates of change of cold-tolerance, although a few observations bearing on this question have been made by Wells (1935) and by Sumner and Doudoroff (1938). The latter problem was now investigated in greater detail.

Limitations of material, time and facilities necessitated the adoption of some single test-period of exposure, for which tolerance limits were to be determined after varying periods of acclimatization to new temperatures. Within certain limits, the longer the period of exposure the more nearly should the results indicate the rate of change of the ultimate or true median tolerance limit. On the other hand, certain practical considerations made the use of a period longer than 24 hours undesirable, and this period was adopted.

Each test lot consisted of ten specimens, carefully selected with regard to size. The specimens varied in length between 5.6 cm. and 7.5 cm., and the mean length in each lot was 6.3 to 6.4 cm. Determinations of heat-tolerance were made in running water, and those of cold-tolerance in standing (aerated) water. The acclimatization temperatures used were 26°, 14° and, in a few experiments, 20°. The specimens were collected in the fall of 1940 and held at current sea-water temperatures. Some were transferred to 26° on October 7, some to 14° on November 16, and some to 20° on December 3.[9] After having been held at these respective (first) acclimatization temperatures for at least 42 days, lots of these specimens were transferred at convenient intervals of time to new (second) acclimatization temperatures. They were held in wire-screen cages immersed in the constant-temperature

[9] The sea-water temperatures on these dates were 19.7°, 17.3° and 16.5°, respectively.

TABLE III

*Per cent survival after 24 hours at various test temperatures and the estimated 24-hour median tolerance limits (T_m) of Girella, which had been acclimatized to various initial (first) temperatures and subsequently subjected for varying periods to other (second) acclimatization temperatures. The percentages are based on 10 specimens, except those marked *, which are based on 20 specimens. Where no second temperature is indicated, the fishes were transferred to the test temperature directly from the first acclimatization temperature. Tolerance limits marked (u) are upper, while the rest are lower median tolerance limits.*

Date of test	First acclim. temp.	Days at first temp.	Second acclim. temp.	Days at second temp.	Test temp.	Per cent surviving 24 hours	T_m (24-hour)
11/18/40	26°	42	—	0	11°	90	10.2°
11/20/40	"	44	—	0	10°	40	
11/25/40	"	49	14°	0.42	10°	80	9.3°
11/28/40	"	52	"	0.42	9°	40	
11/21/40	"	44	"	1	9°	100	8.4°
11/23/40	"	46	"	1	8°	20	
11/27/40	"	48	"	3	8°	90	7.2°
11/24/40	"	44	"	3	7°	40	
12/ 2/40	"	50	"	6	7°	100	6.4°
11/30/40	"	48	"	6	6°	20	
12/10/40	"	52	"	12	6°	90	5.5°
12/ 8/40	"	50	"	12	5°	10	
12/16/40	"	50	"	20	5°	80	4.4°
12/18/40	"	52	"	20	4°	30	
12/20/40	"	42	"	32	5°	90	4.3°
12/18/40	"	42	"	30	4°	30	
1/ 7/41	"	42	"	50	5°	100	4.2°
1/ 5/41	"	42	"	48	4°	40*	
12/11/40	"	65	—	0	11°	100	10.2°
12/ 9/40	"	63	—	0	10°	40	
12/13/40	"	65	20°	2	9°	90	8.2°
12/14/40	"	66	20°	2	8°	40	
12/31/40	14°	45	—	0	4°	60	3.8°
1/ 2/41	"	47	—	0	3°	0	
1/13/41	"	58	26°	0.15	5°	70	4.7°
1/14/41	"	59	"	0.15	4°	0	
1/10/41	"	55	"	0.42	6°	70	5.7°
1/12/41	"	57	"	0.42	5°	10	
1/ 7/41	"	51	"	1	7°	80	6.6°
1/ 4/41	"	48	"	1	6°	0	
1/ 8/41	"	51	"	2	8°	100	7.3°
1/ 8/41	"	51	"	2	7°	30	
1/ 9/41	"	51	"	3	8°	60	7.8°
1/ 7/41	"	49	"	3	7°	0	
1/12/41	"	51	"	6	9°	90	8.5°
1/14/41	"	53	"	6	8°	10	
1/20/41	"	53	"	12	10°	90	9.4°
1/18/41	"	51	"	12	9°	20	
1/28/41	"	53	"	20	10°	55*	9.9°
1/29/41	"	54	"	20	9°	0	

TABLE III—*Continued*

Date of test	First acclim. temp.	Days at first temp.	Second acclim. temp.	Days at second temp.	Test temp.	Per cent surviving 24 hours	T_m (24-hour)
1/31/41	14°	45	26°	31	10°	65*	9.8°
2/ 1/41	"	45	"	32	9°	0	
2/11/41	"	45	"	42	10°	50	10.0°
1/18/41	"	63	—	0	5°	100	4.2°
1/16/41	"	61	—	0	4°	40	
2/ 4/41	"	80	20°	0.42	5°	80	4.6°
2/ 6/41	"	82	"	0.42	4°	0	
1/20/41	"	63	"	2	6°	100	5.5°
1/22/41	"	65	"	2	5°	0	
2/ 1/41	"	69	"	8	7°	100	6.3°
1/31/41	"	68	"	8	6°	30	
1/16/41	20°	44	—	0	7°	65*	6.8°
1/20/41	"	48	—	0	6°	0	
2/ 1/41	"	60	14°	0.42	7°	100	6.2°
1/30/41	"	58	"	0.42	6°	40	
1/20/41	"	46	"	2	6°	100	5.4°
1/23/41	"	49	"	2	5°	10	
2/ 4/41	"	55	"	8	5°	100	4.5°
2/ 6/41	"	57	"	8	4°	0	
1/25/41	"	51	26°	2	9°	100	8.5°
1/27/41	"	53	26°	2	8°	0	
2/ 2/41	"	61	—	0	7°	50	7.0°
11/20/40	26°	44	—	0	31°	100	31.8° (*u*)
11/18/40	"	42	—	0	32°	40	
11/22/40	"	45	14°	1	32°	80	32.4° (*u*)
11/26/40	"	49	"	1	33°	0	
11/24/40	"	45	"	3	32°	80	32.4° (*u*)
11/27/40	"	48	"	3	33°	0	
12/23/40	"	42	"	35	30°	90	30.8° (*u*)
12/21/40	"	42	"	33	31°	40	
12/ 1/40	"	55	—	0	32°	50	32.0° (*u*)
1/ 1/41	14°	46	—	0	30°	70	30.5° (*u*)
1/ 6/41	"	51	—	0	31°	30	
1/ 8/41	"	52	26°	1	31°	100	31.7° (*u*)
1/10/41	"	54	"	1	32°	30	
1 15/41	"	57	"	3	31°	100	31.8° (*u*)
1/13/41	"	55	"	3	32°	40	
2/ 4/41	"	45	"	35	31°	100	31.7° (*u*)
2/ 2/41	"	45	"	33	32°	30	

tanks. Determinations of upper and lower 24-hour median tolerance limits were made with specimens taken directly from the first acclimatization temperatures, and with specimens which had been held for varying periods at the second temperatures. The experimental data and the estimated 24-hour median tolerance limits are presented in Table III. The tolerance limits are plotted against the duration of acclimatization to the second temperatures in Figures 3 and 4.

The difference between the upper limits of tolerance of specimens conditioned to 14° and to 26° was small (1.4°). With respect to the rate of change of heat-tolerance, the results obtained with Girella (Figure 3) are essentially in agreement with those which had been obtained with other species by other methods in earlier investigations. The 14°-conditioned fishes gained heat-tolerance rapidly after transfer to 26°. Acclimatization was apparently complete after about one day, the upper tolerance limit remaining constant thereafter.[10] On the other hand, the 26°-conditioned fishes showed no such rapid loss of heat-tolerance at 14°. Indeed, after one day they appeared to be somewhat more resistant than before, and this apparently increased resistance persisted for three days.[11] After 34 days at 14°, however, their resistance was considerably lower and did not differ significantly from that of fishes initially acclimatized to this temperature.

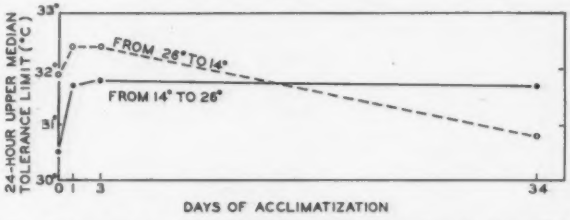

FIG. 3. Rate of change of heat-tolerance with acclimatization: The 24-hour upper median tolerance limits of Girella, initially acclimatized to 14° (solid circles) and to 26° (empty circles), in relation to the duration of acclimatization to new temperatures. The initial (first) and the new (second) acclimatization temperatures are indicated on each curve. Days of acclimatization (abscissas) refer to time at the second (new) temperatures only. The initial tolerance limit (0 days) of 26°-conditioned specimens is the mean of two determinations. Data from Table III.

The data on the rate of change of cold-tolerance are more detailed. The smooth curves which it was possible to fit to the experimental points (Figure 4) will be referred to as "acclimatization curves" of cold-tolerance. Although the curves are not identical in form, no very striking difference is to be noted between the rates of change of cold-tolerance following a corresponding rise and fall of temperature, such as was observed in the case of heat-tolerance. The tolerance limits of fishes transferred from 14° to 26° for acclimatization periods of 0.42 to 20 days may be shown to fall very nearly in a straight line when plotted

[10] Later experiments, which need not be presented in detail, indicated, however, that in two hours complete or even pronounced acclimatization did not occur.

[11] An indication of a similar slight gain of resistance was observed by Sumner and Doudoroff (1938) in Gillichthys. Its significance is too uncertain to warrant discussion.

against the logarithm of the duration of acclimatization. After 20 days at 26° complete acclimatization (i.e., a constant 24-hour lower median tolerance limit) was apparently achieved. The converse acclimatization curve of fishes transferred from 26° to 14° does not show the above logarithmic relationship. Acclimatization was somewhat slower during the first day, but, the curve being somewhat flatter, complete acclimatization again appears to be achieved in 20 days.

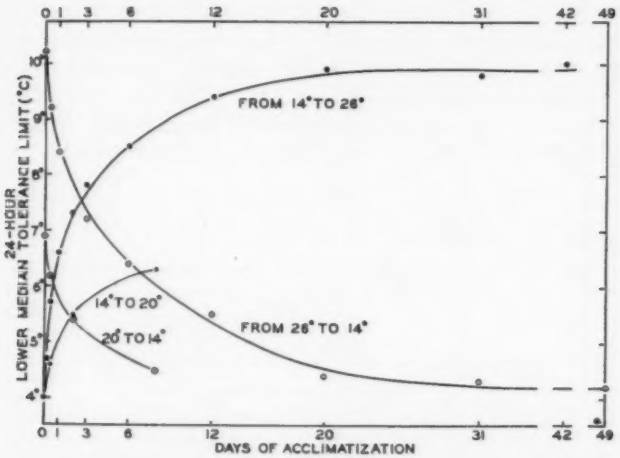

FIG. 4. Rate of change of cold-tolerance with acclimatization (acclimatization curves): The 24-hour lower median tolerance limits of Girella, initially acclimatized to 14° (solid circles), 20° (dotted circles) and 26° (empty circles) in relation to the duration of acclimatization to new temperatures. The initial (first) and the new (second) acclimatization temperatures are indicated on each curve. Points obtained with fishes transferred from 14° to 26° and to 20° are indicated by large and by small solid circles, respectively. Days of acclimatization (abscissas) refer to time at the second (new) temperatures only. The initial tolerance limit (0 days of acclimatization) is in each case a mean of two determinations. Data from Table III. The single determinations with fishes transferred for two days from 20° to 26° and vice versa are not plotted.

To what extent are the above acclimatization curves applicable to and descriptive of changes of cold-tolerance which result from smaller changes of temperature, such as may occur in nature? Although the available data for smaller changes (i.e., 14° to 20° and vice versa, etc.) are not complete, since complete acclimatization has not in any case been achieved, a satisfactory comparison can be made with corresponding portions of the curves obtained with larger temperature changes. The relationships can be illustrated by computing the fraction (per-

centage) of complete acclimatization achieved in each case after given periods (0.4, 2 and 8 days) of acclimatization to the second temperatures. The 24-hour lower median tolerance limits for fishes initially acclimatized to 26°, 20° and 14° and transferred directly to the test temperatures were 10.2°, 6.9° and 4.0° respectively. These values are means of the results of two experiments with each. They may, presumably, be taken to represent the tolerance limits after complete acclimatization. The percentages given in Table IV were calculated

TABLE IV

*Extent of acclimatization, expressed as per cent of total change of cold-tolerance, achieved in varying periods of acclimatization to new (second) temperatures after various changes of acclimatization temperature. (Values marked * were obtained by interpolation.)*

Acclim. temperatures		Per cent acclimatization achieved in:		
Initial (first)	New (second)	10 hours	2 days	8 days
14°	20°	21%	52%	79%
20°	14°	24%	52%	83%
14°	26°	27%	53%	79%*
26°	14°	16%	40%*	69%*
20°	26°		48%	
26°	20°		61%	

on this basis. The corresponding values in this table, that is, values for the same period of acclimatization, show no very striking differences and most show close agreement. In other words, the fraction of the total change of cold-tolerance (complete acclimatization) which occurs in any given time does not vary greatly with the direction or the magnitude of the total change. The somewhat low values obtained consistently with fishes transferred from 26° to 14° may be due to a certain amount of cold-injury, resulting from the sudden cooling to a temperature only slightly above their limit of tolerance. Such injury, while insufficient to cause visible distress, may account for the observed delay in the increase of resistance to further cooling. Gradual recovery, with adjustment to the new temperature, would account for the fact that complete acclimatization was not appreciably delayed, having been achieved in about 20 days, as in the case of the reverse change. Nevertheless, it appears that, at least for any moderate temperature change within the normal temperature range, the course of the acclimatization process (change of cold tolerance) can be described with reasonable accuracy as follows: about 50 per cent of the acclimatization

occurs in two days, and complete acclimatization is achieved in about 20 days, regardless of whether the temperature is raised or lowered.

This rate differs markedly from that reported by Mellanby (1939) for the change of chill-coma temperature in insects. This author usually obtained complete acclimatization in less than 20 hours, and at most in two or three days.

Discussion

It is uncertain to what extent temperatures may determine the distribution of species by virtue of their direct lethality. Non-lethal temperatures may be unsuitable for normal development, growth and activity, or for the activation of reproductive processes, or they may increase susceptibility to disease (e.g., see Staff, 1926). Furthermore, temperature may be a guiding stimulus in the movements of migratory fishes (Danois, 1924; Eggvin, 1937; Ward, 1921). Temperature selection by active fishes has also been demonstrated experimentally (Doudoroff, 1938), but the correspondence of these reactions to those under natural conditions is questionable. Since the distribution of many fishes may be confined to a narrower temperature range than that which is compatible with life, and they are not necessarily ever called upon to withstand temperatures near both their upper and lower limits of tolerance, it is interesting to compare the relative importance of lethal high and low temperatures as potential sources of danger to fishes in their natural habitats and, accordingly, as factors which may limit dispersal. While the opinion that fishes are more sensitive to warming than to cooling has sometimes been expressed or implied, it will be shown that injury by chilling deserves at least as much attention, as a limiting factor in the distribution of marine fishes, as heat-injury. This may not apply to fishes which are normally exposed in their habitat to temperatures at or near the freezing-point of their medium and are adapted to withstand them. Highest possible environmental temperatures are not so sharply defined, although in the open ocean 32° appears to be approximately the upper limit.

The destruction of marine fishes in large numbers in their natural habitat, apparently ascribable directly to unusual temperatures, has been reported repeatedly (Verrill, 1901; Storey and Gudger, 1936; and citations; Miller, 1940; Gunter, 1941). All such reports which have come to the writer's attention deal with destruction by extreme cold and not by heat. This circumstance already suggests a greater importance of lethal cold as a real hazard and as a factor limiting dispersal. Furthermore, of marine fishes which normally live at or near the surface, where temperatures are most variable, the more active and

sensitive ones may be able to escape abnormally high surface temperatures, but not low temperatures,[12] by means of a relatively short vertical migration.

In considering the experimental evidence the previous history of the experimental animals and the temperatures which are normal in their habitat must be taken into account. Maurel and Lagriffe (1899c) apparently failed to consider these factors, and hence their conclusions are of little value. Whether any given temperature should be regarded as more or less extreme than another depends upon its relationship to temperatures at which the fishes in question normally live. The mean surface temperature in the habitat from which the Girella were obtained is about 17°. The mid-value of the average yearly range (i.e., 13.3° to 22.0°), which for present purposes is also a significant point of reference, is 17.7°. Thus a temperature of 17.5° may be regarded as more or less typical of the habitat of young Girella in this locality. Specimens acclimatized to this temperature may be regarded as "normal," and temperatures equally removed from it as equally extreme or atypical. The limits of tolerance of normal Girella can be estimated by interpolation. Referring to Figure 5, the upper

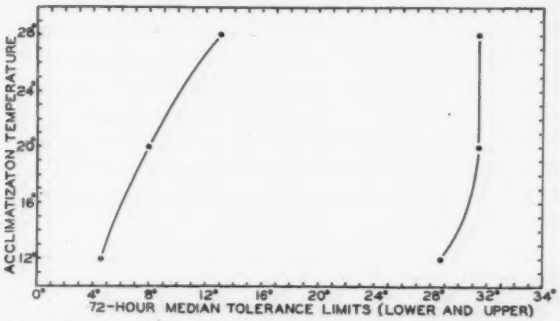

FIG 5. Relation between the temperature of previous acclimatization and the upper and lower 72-hour median tolerance limits. Curves fitted for approximate interpolation. Data from Tables I and II.

and lower median tolerance limits for 72 hours of exposure (the longest period for which complete data are available) corresponding to an acclimatization temperature of 17.5° are found to be about 31° and 6.8°, respectively. Thus the maximal rise of temperature tolerated for three days is by 2.8° greater than the maximal drop, and the lower limit of tolerance is, therefore, less extreme than the upper limit. With

[12] Unlike that of fresh water, the density of sea-water increases with decreasing temperature down to the freezing point.

longer periods of exposure the difference would be still greater. It seems safe to conclude that the fishes are no more resistant to cooling or to extreme low temperatures than they are to warming or to extreme high temperatures.

In nature fishes are not subject to such sudden changes of temperature as have just been considered. That changes of resistance to cold as well as to heat occur with gradual acclimatization has been amply demonstrated. In evaluating their significance, both their magnitude and their rate must be considered. The magnitude of the observed difference of tolerance resulting from a given difference between acclimatization temperatures was variable. The change of heat-tolerance was small in comparison with that of cold tolerance, especially because Girella, unlike other species which have been studied, showed no appreciable increase of heat-tolerance with acclimatization to temperatures above 20°. Possibly Girella could with gradual acclimatization survive cooling to as extreme a temperature as that to which it can be warmed. This, however, is uncertain, and the rise of temperature tolerated by specimens conditioned to the average yearly maximum (22°) is still greater than the drop tolerated by specimens acclimatized to the average yearly minimum (13.3°). This can be deduced from Figure 5. While few generalizations can be made it appears that the change of tolerance, both to heat and to cold, produced by a given change of acclimatization temperature is always considerably smaller than the latter change, and when the results obtained with long periods of exposure only are considered, it tends to decrease as the acclimatization temperature approaches the limit of tolerance.

The range of temperatures tolerated by "normal" Girella is greater than that to which fishes are exposed in the locality in which the specimens were taken. Therefore, the survival value of acclimatization is not obvious in this case. However, acclimatization may make possible the dispersal of a species over a wider range. Furthermore, much greater seasonal variations of surface temperature (i.e., as great as 20° to 28° C.) occur in some localities. Finally, the observed changes of resistance may be only more or less incidental manifestations of more fundamental adjustments of organisms to changes of environmental temperature, involving a general increase of vitality (resistance to disease, etc.) at non-lethal temperatures.

The physiological mechanism of the changes of resistance to heating and to chilling is not known. A change in the degree of saturation and, accordingly, of the melting and solidification points of protoplasmic lipoids has been suggested as a possible factor (Heilbrunn, 1937; Bělehrádek, 1935). The observations that in Girella a large change

of cold-tolerance could be obtained without a corresponding change of heat-tolerance, and that the rates of change of resistance to heat and to cold are quite different, indicate that these two changes are more or less independent of one another. Further investigation of the relative rates of physiological changes which occur with acclimatization (see also Sumner and Doudoroff, 1938) should prove to be of value in establishing which of these may be closely related and perhaps in revealing their mechanisms.

The ecological significance of the rate of acclimatization obviously depends upon the rate of fluctuation of environmental temperatures. In Girella even the relatively slow acclimatization to cold was complete after about 20 days. If this rate is typical, acclimatization should keep pace with seasonal temperature variations. However, large temperature fluctuations in marine environments resulting from the displacement of water masses, etc., may be quite rapid.[13] The gain of heat-tolerance of fishes apparently occurs very rapidly, while its loss on cooling is slow. The survival value of this relationship is obvious. Heat acclimatization will keep pace with a fairly rapid rise of temperature, and in an unstable (eurythermal) environment repeated brief sojourns at high temperatures may result in an increase of tolerance. On the other hand, acclimatization to cold in Girella was relatively slow, and the gain of cold-tolerance no more rapid than its loss. If this relationship is typical,[14] cold acclimatization should be relatively ineffective as a protective mechanism against occasional extreme temperatures in an environment in which fluctuations are rapid. These relationships can be advanced as further evidence that cold as a lethal agent may be more important as a factor limiting distribution than lethal heat.

The great sensitiveness of Girella and of other marine fishes to low temperatures or to cooling was further indicated by their reactions to temperature gradients (Doudoroff, 1938), in which the avoidance of low temperatures was much more pronounced than that of high temperatures. The temperatures most frequently selected by Girella which had been held at normal seasonal temperatures were 26° to 27°, i.e., much nearer their upper than their lower limits of tolerance. The fact that the reactions, just as heat-tolerance, were altered much more by acclimatization to very low temperatures than to high temperatures (Doudoroff, 1938, Figure 4) indicates a possible relationship between heat-resistance and selection in the gradient. However, the relative

[13] E.g., see Parr (1933), who reports changes of surface temperature in open water as great as 11° C. in two days.

[14] Some results of Sumner and Doudoroff (1938) indicate that it probably holds also for Gillichthys (see alternation experiments, loc. cit., pp. 421–422).

rates of acclimatization to higher and lower temperatures, as indicated by the reactions (Doudoroff, 1938, Figure 5), were more similar to the corresponding rates of change of cold-resistance than of heat-resistance.

Summary

The resistance of young *Girella nigricans* (Ayres) to low and to high temperatures and the changes of tolerance produced by acclimatization are examined in relation to temperatures in the natural habitat of the fishes.

The fishes were killed by moderately low temperatures well above 0° C., the lower limits of temperature tolerance being no more extreme, in comparison with normal environmental temperatures, than the corresponding upper limits.

The relationship between the time (duration) of exposure to the test temperatures and the highest or lowest temperatures tolerated by 50 per cent of the experimental animals (upper or lower "median tolerance limits") is representable by smooth time-temperature curves of tolerance.

Acclimatization to different temperatures has a pronounced influence upon subsequent resistance to cold (chilling), as well as to heat, and upon the form of the respective time-temperature curves of tolerance.

Experiments on the rate of acclimatization corroborate the conclusion, based on earlier observations, that heat-resistance in fishes is gained rapidly after a rise of temperature and lost slowly after cooling.

Acclimatization to cold (i.e., increase of resistance to chilling) is relatively slow, and cold-resistance is lost no more slowly than it is acquired. After any given rise or fall of environmental temperature within the normal range, about 50 per cent of the total resulting change of cold-tolerance occurs in two days, and complete acclimatization (i.e., constant cold-tolerance) is apparently achieved in about twenty days.

Injury by chilling is no less important as a possible limiting factor in the distribution of marine fishes than heat-injury.

Literature Cited

Bělehrádek, J., 1935. Temperature and living matter. *Protoplasma-Monographien*, **8**: 277 pp. Berlin. Gebruder Borntraeger.

Binet, L. and G. Morin, 1934. Action de la chaleur sur les poissons. *Jour. de Physiol. et de Pathol. Générale*, **32**: 372–379.

Bodenheimer, F. S. and H. Z. Klein, 1930. Über die Temperaturabhängigkeiten von Insekten. II. Die Abhängigkeit der Aktivität bei der Ernteameise *Messor semirufus* E. André von Temperatur und anderen Factoren. *Zeitschr. f. vergl. Physiol.*, **11**: 345–385.

BORODIN, N. A., 1934. The anabiosis or phenomenon of resuscitation of fishes after being frozen. *Zool. Jahrb., Abt. f. allg. Zool. u. Physiol.*, **53**: 313–341.

BRITTON, S. W., 1924. The effects of extreme temperatures on fishes. *Am. Jour. Physiol.*, **67**: 411–421.

DANOIS, E. LE, 1924. Étude hydrologique de l'Atlantique-nord. *Ann. l'Inst. Océanogr.*, n.s., **1**: 1–52.

DOUDOROFF, P., 1938. Reactions of marine fishes to temperature gradients. *Biol. Bull.*, **75**: 494–509.

EGGVIN, J., 1937. Oceanographical conditions in North-Norway connected with the cod fisheries (Summary in English). *Fiskeridirektoratets Skrifter (Report Norw. Fish. and Mar. Investig.)*, **5**: 51–52.

FOX, H. M., 1939. The activity and metabolism of poikilothermal animals in different latitudes. *Proc. Zool. Soc.*, **109**: 141–156.

GUNTER, G., 1941. Death of fishes due to cold on the Texas coast, January 1940. *Ecology*, **22**: 203–208.

HATHAWAY, E. S., 1927. Quantitative study of the changes produced by acclimatization in the tolerance of high temperatures by fishes and amphibians. *Bull. Bureau of Fisheries*, **43**, II: 169–192.

HEILBRUNN, L. V., 1937. An outline of general physiology. Philadelphia. W. B. Saunders Co., 603 pp.

HÖRSTADIUS, S., 1925. Temperaturanpassung bei den Eiern von *Paracentrotus lividus* Lk. *Biologia Generalis*, **1**: 522–536.

HUNTSMAN, A. G., 1926. The comparative thanatology of marine animals. *Trans. Roy. Soc. Canada*, **20**, V: 187–208.

HUNTSMAN, A. G. AND M. I. SPARKS, 1924. Limiting factors for marine animals. 3. Relative resistance to high temperatures. *Contrib. Canadian Biol.*, n.s., **2**: 97–114.

JORDAN, D. S., B. W. EVERMANN AND H. W. CLARK, 1930. Check list of fishes and fish-like vertebrates of North and Middle America north of the northern boundary of Venezuela and Colombia. *Report of the U. S. Commissioner of Fisheries, 1928*, II, **1055**: 670 pp. Washington, Government Printing Office.

LOEB, J. AND H. WASTENEYS, 1912. On the adaptation of fish (Fundulus) to higher temperatures. *Jour. Exp. Zool.*, **12**: 543–557.

LUYET, B., 1938. Sur la survie des poissons plongés dans l'air liquide. *C. R. Soc. Biol.*, **127**: 788–790.

LUYET, B. AND P. M. GEHENIO, 1938. The lower limit of vital temperatures—a review. *Biodynamica*, **33**: 1–92.

MAUREL AND LAGRIFFE, 1899a. Determination et action des plus hautes températures compatibles avec la vie de certains poissons. *C. R. Soc. Biol.*, **51**: 797–800.

MAURELL AND LAGRIFFE, 1899b. Determination et action des plus basses températures compatibles avec la vie de certains poissons. *C. R. Soc. Biol.*, **51**: 875–878.

MAUREL AND LAGRIFFE, 1899c. Action comparée de la chaleur et du froid sur certains poissons. *C. R. Soc. Biol.*, **51**: 915–918.

MELLANBY, K., 1939. Low temperature and insect activity. *Proc. Roy. Soc. London*, B, **127**: 473–487.

MELLANBY, K., 1940. Temperature acclimatization in amphibia. *Jour. Physiol.*, **98**: 27–28 P (*Proc. Physiol. Soc.*).

MILLER, E. M., 1940. Mortality of fishes due to cold on the southeastern Florida coast, 1940. *Ecology*, **21**: 420–421.

OGLE, C. AND C. A. MILLS, 1933. Animal adaptation to environmental temperature conditions. *Am. Jour. Physiol.*, **103**: 606–612.

PARR, A. E., 1933. A geographic-ecological analysis of the seasonal changes in temperature conditions in shallow water along the Atlantic coast of the United States. *Bull. Bingham Oceanogr. Collection*, **4**, (3): 90 pp.

PAYNE, N. M., 1926a. The effect of environmental temperatures upon insect freezing points. *Ecology*, **7**: 99–106.

PAYNE, N. M., 1926b. Freezing and survival of insects at low temperatures. *Quart. Rev. Biol.*, **1**: 270–282.

REGNARD, P., 1895. Action des très basses températures sur les animaux aquatiques *C. R. Soc. Biol.*, **2**: 652–653.

SCHMIDT, P. J., G. P. PLATONOV AND S. A. PERSON, 1936. On the anabiosis of fishes in supercooled water. *C. R. Acad. Sci. U.R.S.S.*, **3**: 305–308.

STAFF, F., 1926. Maladie des narines de la carpe. Contribution sur la physiologie et pathologie du sommeil hivernal. *Internat. Rev. de Hydrobiol.*, **14**: 146–159, 312–321.

STOREY, M., 1937. The relation between normal range and mortality of fishes due to cold at Sanibel Island, Florida. *Ecology*, **18**: 10–26.

STOREY, M. AND E. W. GUDGER, 1936. Mortality of fishes due to cold at Sanibel Island, Florida, 1886–1936. *Ecology*, **7**: 640–648.

SUMNER, F. B. AND P. DOUDOROFF, 1938. Some experiments on temperature acclimatization and respiratory metabolism in fishes. *Biol. Bull.*, **74**: 403–429.

SUMNER, F. B. AND M. C. SARGENT, 1940. Some observations on the physiology of warm spring fishes. *Ecology*, **21**: 45–54.

U. S. Weather Bureau, 1938. Atlas of climatic charts of the oceans. *W. B. no.* **1247**: 130 pp.

VERRILL, A. E., 1901. A remarkable instance of the death of fishes at Bermuda in 1901. *Am. Jour. Sci.*, 4, **12**: 88.

WARD, H. B., 1921. Some of the factors controlling the migration and spawning of the Alaska red salmon. *Ecology*, **2**: 235–254.

WEIGMANN, R., 1929. Die Wirkung starker Abkühlung auf Amphibien und Reptilien. *Zeitschr. f. wissenschaft. Zool.*, **134**: 641–692.

WEIGMANN, R., 1930. Weitere Untersuchungen über die Kältebeständigkeit poikilothermer Wirbeltiere. *Zeitschr. f. wissenschaft. Zool.*, **136**: 195–209.

WEIGMANN, R., 1936. Zur Kältebeständigkeit poikilothermer Tiere, Untersuchung an Schnecken und Fischen. *Biol. Zentralblatt*, **56**: 301–322.

WELLS, N. A., 1935. Variations in the respiratory metabolism of the Pacific killifish *Fundulus parvipinnis* due to size, season, and continued constant temperature. *Physiol. Zool.*, **8**: 318–336.

WHITNEY, R. J., 1939. Thermal resistance of mayfly nymphs from ponds and streams. *Jour. Exper. Biol.*, **16**: 374–385.

SOME EFFECTS OF HOMOTYPIC EXTRACTS ON THE RATE OF CLEAVAGE OF ARBACIA EGGS

W. C. ALLEE, A. J. FINKEL, H. R. GARNER, G. EVANS AND R. M. MERWIN [1]

(Marine Biological Laboratory, Woods Hole, and Department of Zoology, University of Chicago)

In a series of papers in 1937, Allee and Evans reported that under a variety of experimental conditions eggs of the purple sea urchin, *Arbacia punctulata*, show an accelerated cleavage rate when in relatively dense, as contrasted with relatively sparse, populations. Frank and Kurepina (1930) had obtained similar indications with a sea urchin from the Murmansk coast of Russia, and Maxia (1933), with *Paracentrotus lividus*, when tested at or near the beginning of swimming, had found most rapid development with 40 to 65 eggs in seven drops of sea water. These European workers interpreted the observed results in terms of mitogenetic rays.

Sugiyama (1938), with eggs of still other sea urchins, *Anthocidaris crassipina*, *Pseudocentrotus depressus*, and *Strongylocentrotus pulcherrimus*, also found an optimum density for most rapid cleavage. In his work this optimum came with 20 to 30 eggs in five to ten drops of sea water. He does not record his method of testing for causal factors but he does say that mitogenetic rays are not involved.

This paper deals with a number of possible underlying causes for the acceleration of early cleavage in Arbacia eggs; in this connection the role, conjectural at best, of mitogenetic rays has been ignored. In a preliminary survey, Allee and Evans (1937*b*) found that the observed acceleration of cleavage rate in the denser populations of Arbacia eggs was not to be ascribed to differential temperatures externally imposed, nor to differential hyper- or hypotonicity, nor to contamination with coelomic fluid or fragmented eggs. Further, while the denser populations lowered the pH significantly, an indication of a possible role of carbon dioxide, appropriate tests with varying amounts of carbon dioxide up to that sufficient to produce the same pH observed in dense populations, had no stimulating effects on the rate of cleavage. In this connection Smith and Clowes (1924) and Haywood and Root (1930) found that slight increases in carbon dioxide tension retarded cleavage

[1] The first two authors were actively engaged in these and related experiments for three summers or more; the last three each worked for a single season.

of Arbacia eggs. Cleavage was also retarded rather than accelerated by placing fertilized eggs which had been well washed, in water in which other eggs had stood, whether these were unfertilized, recently fertilized, or undergoing cleavage. This last set of results taken with those of Springer (1922) and Peebles (1929), indicates that the presence of accelerating substances or conditions shown by the more rapid cleavage in the denser populations is masked or inhibited when the entire conditioned medium is present but the developing eggs are lacking.

A series of experiments was carried out at the Marine Biological Laboratory at Woods Hole during the summers of 1939, 1940, and 1941 in an effort to examine this problem of possible cleavage-promoting substances, and to this end a number of extracts of developing Arbacia eggs were prepared.

Preliminary Extracts

Peebles (1929) made an extract of Arbacia plutei by putting them in 90 per cent alcohol or 50 per cent acetone; "then the mixture without grinding the plutei was evaporated to dryness, and to a given quantity sea water was added" (p. 183). Such preparations retarded the growth of plutei. "If however, the fats are removed from the acetone extract, and a water solution made from the residue there is evidence that the inhibiting effect is removed."

We have made acetone extracts as follows: Eggs were washed four times, fertilized, allowed to pass third cleavage, and centrifuged lightly to concentrate. The eggs were shaken up in 50 per cent reagent grade acetone and let stand in different preparations from one to 22 hours. They were then centrifuged and the supernatant acetone was removed. In one preparation 0.7 cc. of eggs was treated with 10 cc. of 50 per cent acetone. For this extract an extract-control was made by adding 10 cc. of such acetone to 0.2 cc. of sea-water; the resulting solution was treated as was the egg extract. In both, one fraction was dried to constant weight immediately and the other after having been treated with ether to remove the ether soluble materials was similarly dried. Each fraction was taken up in sea water before being used in the experiments.

The procedure followed in assaying the extract was essentially the same as that used by Allee and Evans (1937*b*). With appropriate modifications of which the important ones will be mentioned later the same general technique was used in all the assays reported in this paper. All experiments were carried on in a room with north exposure; the windows were closed to avoid excess evaporation and sudden

changes in temperature. The customary precautions regarding the handling of the sea urchins, their sexual products, and the glassware were scrupulously observed. As a matter of course, within any experiment, all the eggs came from one female. Freshly filtered sea water was used throughout. The eggs were fertilized in about 150 cc. of sea water in a finger-bowl. After five minutes, portions of the fertilized stock were transferred by means of a dropping pipette to Syracuse watch dishes in which extracts of the appropriate dilutions had been previously placed. Adjacent dishes contained the extract or the sea-water controls. After three minutes in the Syracuse dishes, the eggs were transferred, in mildly sparse suspensions, to small shell vials by means of a haemocytometer pipette. These vials were placed in rows and columns in a brass holder. When the transfer was complete, each vial contained 20 cu. mm. of sea water, treated or untreated, plus the eggs contained in this volume. The time occupied by the transfer from Syracuse dishes to the vials ranged from 8.5 to 17.3 minutes, and averaged 11.7 minutes; the entire period from fertilization to completion of transfers averaged 19.6 minutes. In most experiments 48 vials were used.

Precautions were taken in the design of the experimental set-up to prevent prejudice in favor of either the control or the extract insofar as position in the holder or, more important, the order in which the eggs were transferred to the vials was concerned. The metal holder containing the vials was placed in a moist chamber which in turn was placed in a cold cabinet where the temperature was kept at 19° C. in order to retard the progress of cleavage so that the eggs might be in contact with the extract for a longer time.

Rows of eggs were killed during the progress of second cleavage by the addition to the vials of one per cent formalin in sea water at 0.5, 1, 1.5, or 2 minute intervals, depending on the progress of cleavage as observed through a binocular microscope. Great care was taken by means of elaborate washing procedures in cleaning out all possible traces of formalin from the vials before they were used again in an experiment. Allee and Evans (1937*a*) showed that the presence of traces of toxic material produced retardation of cleavage in sparse populations of eggs as contrasted with a reduced effect with denser populations. In the present experiments comparable numbers of eggs were used in experimental and control suspensions and in any event throughout all experiments the presence of toxic material, other than that which may have been an intrinsic part of the accelerating extract itself, was scrupulously avoided.

After this serial killing of all the rows in a given experiment, vials of eggs were washed successively into large depression slides and the eggs were counted under the low power of a compound microscope for one-, two-, and four-celled stages. Throughout the work results were not used when the cleavage fell below 90 per cent; usually it was 95 per cent or more.

Twelve experiments were made with a series of different solutions of the acetone extracts. When the results at or near 50 per cent second cleavage were studied, six per cent more eggs had cleaved in the whole acetone extract than in the accompanying sea water controls. This mean apparent acceleration, when examined for statistical significance by "Student"'s method for paired comparisons, had a P-value of 0.0984.[2] The fat-free portion gave a mean apparent acceleration of 12.1 per cent, but with much wider variation, and the P-value was 0.0941. Since P was greater than 0.05 in both instances, the seeming accelerations lacked statistical validity. When the rate of cleavage of eggs in the acetone extracts was compared with that of those in the extract-controls, the apparent acceleration of 8.3 per cent also lacked statistical significance with $P = 0.1096$. It is evident, then, that such extracts failed to stimulate the rate of cleavage.

Another extract was more elaborately prepared (Figure 1), and the potency of various fractions tested. The extract was made from a large number of eggs which had passed third cleavage. These were centrifuged down to 1.2 cc. and were ground with clean, ignited sea-sand and distilled water; they were again centrifuged to throw down the sand and the larger particles. The resulting red, watery, supernatant liquid was divided into three fractions: A, B, and C. Fraction A was treated with three times its volume of 99.5 per cent acetone and again centrifuged; the supernatant portion was evaporated to dryness at low temperature and was soon thereafter taken up in 10 cc. sea water and tested for its effect on the rate of cleavage in various dilutions. The precipitate was washed twice with distilled water, twice with absolute ethyl alcohol; the precipitate was mixed with 5 cc. ethyl alcohol and the suspension was divided. Half the material was evaporated to dryness in a desiccator, and the dried material was taken up in 10 cc. 0.05 N NaOH at room temperature for eight hours, and then stored in the refrigerator. The other half was washed with ether, dried, washed with 10 cc. distilled water, and then taken up in 10 cc. 0.01 N

[2] Statistical significance is given by the P-value which is calculated by the method of "Student" (1925). This method is designed for the analysis of situations where a small number of cases is involved. A P-value under 0.05 is customarily considered as indicating statistical significance; it is roughly equivalent to two times the standard error.

NaOH and stored in the refrigerator. Both of these fractions were assayed promptly in various concentrations against sea water as well as against comparable NaOH controls.

Fraction *B* had 2.5 times its volume of 95 per cent ethyl alcohol added. After centrifuging, the supernatant liquid was stored in the icebox (for two weeks) after which it was dried in a desiccator, and was later taken up in sea water for assay; this fraction, called by us "*Ba*," will be reported on at length later. The precipitate from fraction *B* was treated essentially as was the precipitate from fraction *A* except

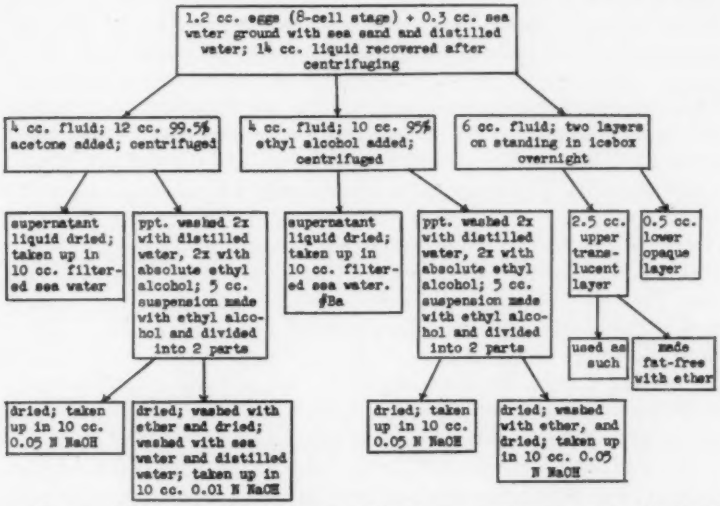
1.2 cc. eggs (8-cell stage) + 0.3 cc. sea water ground with sea sand and distilled water; 1½ cc. liquid recovered after centrifuging
4 cc. fluid; 12 cc. 99.5% acetone added; centrifuged
4 cc. fluid; 10 cc. 95% ethyl alcohol added; centrifuged
6 cc. fluid; two layers on standing in icebox overnight
supernatant liquid dried; taken up in 10 cc. filtered sea water
ppt. washed 2x with distilled water, 2x with absolute ethyl alcohol; 5 cc. suspension made with ethyl alcohol and divided into 2 parts
supernatant liquid dried; taken up in 10 cc. filtered sea water. #Ba
ppt. washed 2x with distilled water, 2x with absolute ethyl alcohol; 5 cc. suspension made with ethyl alcohol and divided into 2 parts
2.5 cc. upper translucent layer
0.5 cc. lower opaque layer
used as such
made fat-free with ether
dried; taken up in 10 cc. 0.05 N NaOH
dried; washed with ether and dried; washed with sea water and distilled water; taken up in 10 cc. 0.01 N NaOH
dried; taken up in 10 cc. 0.05 N NaOH
dried; washed with ether, and dried; taken up in 10 cc. 0.05 N NaOH

FIG. 1. Fractionation of extracts of Arbacia eggs. This paper is largely based on work with extract No. *Ba*. The other fractions failed to show acceleration; see text.

that this time both sub-fractions were taken up in 0.05 N NaOH for assay.

Fraction *C* of the original extract separated into two layers on standing in the icebox overnight. The upper translucent layer was (1) tested as such, and (2) was made fat-free with ether and tested. The lower, opaque layer was diluted in sea water and when tested produced a marked retardation.

The only one of these sub-fractions which gave an indication of producing fairly steady acceleration of cleavage when tested against appropriate controls was the alcohol extract called "*Ba*"; further experimentation centered on this extract.

Alcohol extract

Our problem was not concerned with assaying the effects of various homotypic extracts upon the rate of cleavage of Arbacia eggs; rather it was to find some possible factors which might produce the observed acceleration when populations of sea urchin eggs are relatively crowded as compared with well-scattered populations. We therefore felt justified in focussing attention on that part of the extract which produced acceleration most regularly in the early assays.

The first batches of this extract were made according to the directions given in the preceding pages. Later, a simplified procedure was used. The eggs were prepared for extraction as usual and then a small amount of distilled water was added; sand was no longer used in view of the experience of Grave (1935). The mixture was allowed to stand with occasional stirring for about an hour; by this time the eggs were fairly well broken. Then 95 per cent or absolute ethyl alcohol was added, and after a few minutes the mixture was centrifuged and the supernatant liquid was decanted into a crucible for drying to constant weight, which was accomplished by gentle heat, 40° C. or less.

Small amounts of the dried extract were taken up in sea water to make stock solutions which were stored in the refrigerator. Experience soon showed that even a well-chilled stock solution changed in potency after relatively few hours, and that consistent results could not be expected by the third day. Successive hundredfold dilutions were made from a stock solution, and from each of these dilutions 1.0 cc. was added to a measured amount of sea water which had been placed in a Syracuse dish, and to which was then added a measured amount of suspension of fertilized eggs. In different experiments the latter quantity varied from one to 3 cc.; the total amount in the Syracuse dish ranged from 7 to 10 cc. That portion of the extract which proved to be insoluble was subsequently recovered from the original stock, and was washed, dried and weighed to determine the amount which had presumably gone into solution. The average amount of the dissolved material was .125 mgm. per cc. in the original stock solution. The range of the assay concentrations for the three years was: 8.4×10^{-5} to 8.4×10^{-14} mgm. per cc. Since the spacing of the concentrations within these limits varied somewhat, and since the exact values of dissolved portions were not determined for all the batches of extracts used, the concentrations given in Table I are approximate only and serve to show orders of magnitude rather than precise quantities.

The method of assay during 1939 and a part of 1940 has already been described. For the remainder of the tests cleavage was followed in Syracuse dishes rather than in small vials. The former were set on

a clean towel on the laboratory bench in two or three rows of five each: one row for the experimental extracts and the others for the controls. The latter contained the same amount of sea water and eggs as did the experimental dishes. One control always contained untreated sea water; the other, when present, was a check on the possibility that some foreign material in the solvent, or traces of the solvent itself, might be causing the observed accelerations. This latter control was prepared by making use of the same quantities of all the ingredients and the same treatments used in making the regular alcohol extracts with the important exception that no eggs were used. It was, in fact, an alcohol extract of approximately 0.3 cc. of sea water, plus whatever residues might have been left from treatment with double-distilled water and the laboratory alcohol. An analysis of all the data collected on this point showed no significant difference in the rate of cleavage in the two types of controls.

TABLE I

Summary of analysis of assays of the alcohol extract during the summers of 1939, 1940 and 1941

Approximate concentrations of extract in mgm. per cc.	Mean difference in per cent cleaved between extract and sea-water control at or near 50 per cent second cleavage	Number of experiments	*P* values
8.4×10^{-5}, 10^{-6}	7.56	48	0.0007
8.4×10^{-7}, 10^{-8}	6.1	43	0.0093
8.4×10^{-9}, 10^{-10}	4.97	43	0.0029
8.4×10^{-11}	5.04	32	0.0357
8.4×10^{-12}, 10^{-14}	−1.34	23	not significant

In the more recent assays, the eggs were fertilized as usual in a finger bowl. After five minutes, 3 cc. of egg suspension were added simultaneously to an experimental dish and to its controls. The whole process of distributing eggs into the Syracuse dishes took about half a minute. The eggs were then undisturbed except for occasional examination under a binocular microscope until the time of second cleavage, 65 to 85 minutes later.

Sample lots of the cleaving eggs were killed several times during second cleavage by dropping a few drops of each egg suspension into separate vials which already contained a small amount of 2 per cent formalin in sea water. This method of killing requires due care lest the pipettes become contaminated. The formalin stopped development immediately and the percentage of cleavage at the time of killing was determined by sample counts made during the subsequent few hours.

The results of a typical experiment from 1939 are illustrated in Figure 2. The solid lines show the ascertained segment of the cleavage

curve for two concentrations of the extract: 5×10^{-5} mgm. per cc. (triangles) and 5×10^{-6} mgm. per cc. (squares). The broken lines show the appropriate controls in sea water and their mean. Cleavage in this experiment was 96 per cent. The mean difference in percentage cleaved when the accelerated experimentals were at mid-cleavage was 21 per cent.

During the 1939 season this extract was tested in dilutions that ranged from 5×10^{-5} to 5×10^{-10} mgm. per cc. Acceleration was

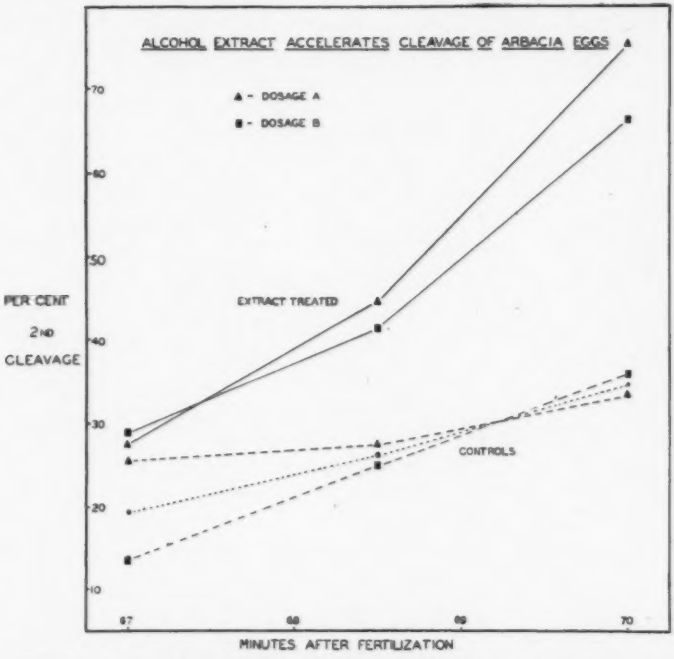

FIG. 2. Acceleration of second cleavage of Arbacia eggs by treatment with homotypic extracts. See text for details.

most effective in the relatively greater concentrations although dilutions as great as 5×10^{-9} mgm. per cc. seemed to show an accelerative influence. In the more effective dosages, in the dilutions just noted, in 21 paired experiments, 99 paired comparisons, of which six are shown in Figure 2, indicated that the treated eggs were ahead of their controls. The differences between the percentages of the eggs which achieved second cleavage averaged 10.59. An analysis for the 99 pairs gave a *P*-value of less than 0.0000001, a highly significant figure.

These data were further analyzed by another method, one of which was used in the previous work by Allee and Evans. In each experiment the percentage of cleavage nearest the 50 per cent mark for the treated eggs was compared with the value for the corresponding untreated eggs; again, the value nearest 50 per cent for the controls was compared with that for their comparable experimental eggs. The average of these two differences was used in the analysis by paired experiments. The formula, then, for the value of the difference is

$$\frac{(50\%\ \text{Exp.—its Control}) + (50\%\ \text{Control—its Exp.})}{2}.$$

Twenty-one such paired experiments of mid-cleavage values showed that the mean percentage difference between extract-treated eggs and their controls was 10.36, with a significant *P*-value of 0.0025.

Although the results in 1939 seemed fairly conclusive and were made the basis for a preliminary report (Allee and Finkel, 1939), we spent parts of two more summers in further work on the subject, and have made some refinement in procedure. Thus, for 1941, cleavage curves were constructed from the data. Where possible, percentage differences were read from the 50 per cent point of second cleavage on the control line vertically to the experimental line, and similarly from the 50 per cent point on the latter line vertically to the control line. The mean of these values was taken to indicate percentage difference at 50 per cent cleavage.

All available results for the three seasons are summarized briefly in Tables I and II. Table I shows mean acceleration for different levels

TABLE II

Comparison of treatments by summing all concentrations of alcohol extracts

Comparison	Mean difference in per cent	*P* values
Extract *vs.* sea-water control	5.952	0.0010
Extract *vs.* extract control	8.218	0.0008
Extract control *vs.* sea-water control	−2.605	0.1050

of dilution of the extract. It will be noted that there is the largest mean difference at the strongest concentrations of the extract and that there is also the greatest statistical significance for these results. Also, in general there is a suggestion of a dilution effect which becomes evident in the weakest concentrations. The differences, while small, are highly significant statistically for the three stronger concentrations. Should we, therefore, have tried still stronger concentrations? The indications are that we should not. The strongest evidence comes from the work of 1940 when the greatest percentage difference (5.8) was found with those concentrations shown in the second and third lines of

Table I, and the greatest statistical significance ($P = 0.0176$) was shown with the concentrations summarized in the third line.

Since the mean percentage difference in acceleration shown by the combined data for the three summers' work varies relatively little between extract strengths of 8.4×10^{-5} and 8.4×10^{-11} mgm. per cc., it is interesting and probably fair to combine all these data and examine their statistical significance. These results are given in Table II. The mean acceleration shown by the eggs in the extracts as compared with others in the sea-water controls is seen to be a trifle under 6 per cent with good statistical likelihood of being significant. Further, the acceleration is over 8 per cent when the extract-treated eggs are compared with others in accompanying extract-controls, with still better statistical significance. The slight retarding tendency of the extract-controls does not approach statistical validity when compared with the results in sea water.

For some purposes it is interesting to examine the results in a different way. If one studies the cleavage curves for the different concentrations of the 59 experiments which met our standards, there are in all 356 parts of these experiments in which it is possible to get valid comparisons between the trends of the partial cleavage curves for experimental and control eggs. Many of these fragments of cleavage curves were too far removed from the 50 per cent level to be in the comparisons given in Table I. Of these 356 cleavage curves, 63.2 per cent showed acceleration of eggs in the extract as compared with those in the accompanying sea-water controls. This percentage is about the same for each of the three summers during which these experiments were made, and for the extracts prepared by each of the three workers who attempted to make them. This percentage of positive experiments is not out of line with the mean acceleration and the statistical significance which we have observed.

Echinochrome

The alcohol extract with which acceleration was obtained was red in color; it had about the same color as that shown by a concentrated mass of eggs. This coloring matter in sea urchin eggs was found by McClendon (1912) to be identical with the previously described pigment echinochrome. Since some echinochrome was obviously present in our extracts, it seemed advisable, during the summer of 1941, to determine just how much was present. It was found that about half the echinochrome in the eggs was carried into the alcohol extracts, and that these extracts when dry were about 25 per cent echinochrome. The next step was to purify some echinochrome and determine whether it had

any effect on the rate of development. The method of purification was essentially that of Ball (1934) and was done by repeated precipitation by ammonium hydroxide from an acid alcohol solution. The final alcohol solution was evaporated and extracted with distilled water and petroleum ether. It was then dried to constant weight before solution in sea water for use in the experiments. A total of 16 experiments conducted in the same manner as those with the alcohol extracts failed to show either acceleration or inhibition by echinochrome in a wide range of concentrations, from approximately 5×10^{-3} mgm. per cc. to 5×10^{-11} mgm. per cc. Hence, the accelerating effects of the alcohol extracts are not a result of the echinochrome they contain.

Discussion

It should be remembered that the present paper records the results of experiments which were made in an attempt to analyze for possible causal factors that produce more rapid cleavage in dense populations of Arbacia eggs as compared with those in sparse populations. It has already been recalled that earlier work (Allee and Evans, 1937*a*, *b*) showed that neither externally imposed temperature nor effects of hyper- or hypotonicity are involved. All of the work of the senior author and his associates supports the statements in the literature that contamination with coelomic fluid, the presence of numbers of broken eggs, the use of water in which unfertilized eggs have stood ("egg water"), or in which fertilized eggs have cleaved ("cleavage water"), and the presence of even slight increases in CO_2 tension, all retard rather than accelerate cleavage of Arbacia eggs introduced into such conditions. Evidently the explanation of the increased rate of cleavage in denser populations is not to be in terms of the action of such contaminations. Neither is there any indication that this density effect can be interpreted in terms of reduced calcium in the sea water, a condition which has been shown to accelerate Arbacia cleavage (Shapiro, 1941).

The amount of acceleration for which explanation is needed was shown by Allee and Evans (1937*a*, p. 224) to average about three minutes at 50 per cent second cleavage in one extended set of experiments in which the conditions were not quite comparable with those used in the present work. Under the conditions then tested the sparse populations of eggs reached the mid-point of second cleavage in 97.67 minutes while the denser populations took 94.72 minutes. This difference of 2.95 minutes is statistically significant ($P = 0.0016$) and represents an acceleration of 3.2 per cent on the horizontal axis of the cleavage curve. The maximum acceleration observed in the work which was reported

in 1937 was on the order of ten minutes in 90 minutes elapsed time after fertilization. In another set of experiments in which the assays were made by counting eggs killed at spaced intervals, as in the present experiments, the mean difference in per cent cleaved at approximately the mid-point of second cleavage was 18 per cent ($P < 0.00001$) (Allee and Evans, 1937*b*, p. 19). By interpolation with tests of the action of copper, to be discussed a little later, in which there was a difference, at the middle of second cleavage, of 18.6 per cent and for which cleavage curves showed a mean difference of 60.8 seconds, we can assume that the time difference observed by Allee and Evans was of the same order. Hence we have to find some accelerating mechanism which will account for a hastening of at least one minute at 50 per cent second cleavage or of at least 18 per cent difference in percentage cleaved, since this was the amount observed in experiments similar to those in which cleavage was hastened by the presence of relatively dense populations of cleaving eggs.

The mean increase in rate of cleavage to the same end point which was produced by the alcohol extract, with which we have been mainly concerned in the present work, was between 6 and 8 per cent (Table II); this means that the mean difference in elapsed time was approximately 20 to 25 seconds at the middle of second cleavage. The extract then produces an acceleration which is 30 to 45 per cent of that observed in the most nearly comparable basic dense-sparse experiments. We have no ground for assuming, however, that any part of the acceleration in the basic experiments was in fact a result of the action of a substance comparable to our extract. The experiments do show that there is in fertilized eggs a readily prepared substance, or substances, which, under appropriate conditions, will produce at least one-third of the increase in the rate of cleavage as does optimal crowding.

While the differences we have observed between extract-treated and control groups of eggs are small, they are highly significant statistically. This significance is primarily a consequence of a fair degree of consistency of the values of these differences throughout a large number of experiments. As might be expected under the circumstances, not all of the experiments yielded positive results, and there were even some runs of experiments which, when averaged together, show no statistically suggestive acceleration. This means either that the accelerating principle in our extract is not very potent or that its activity has been offset in part by the presence of contaminants. The whole situation is complicated by the known variability of Arbacia eggs both as to season and from female to female. We know that the extract as

used is a mixture of unidentified parts and our data give no indication of its potency if purified.

In another series of experiments (Allee, Finkel, and Garner, 1941) it was found that copper, added as $CuCl_2$ to sea water, produced at the optimum concentration of 10^{-13} M a mean acceleration at 50 per cent second cleavage of 69.4 seconds ($P = 0.0004$). In order to check the possibility that copper as a contaminant may have been responsible for the accelerating effects of the extracts, these were analyzed for the presence of copper. The analysis was done by the standard method of colorimetric determination with sodium diethyl-dithio-carbamate. The details of the analysis are described in another paper in greater detail (Finkel, Allee, and Garner, 1942). Suffice it to say at this point that the extracts analyzed showed a mean copper content of 11.29×10^{-6} mgm. per mgm. of dried extract material. Since our records show that an average of 10.68 mgm. of extract, soluble and insoluble, was taken up in the basic stock solutions, such stocks of 10 cc. would accordingly contain 12.05×10^{-6} gm. of copper per liter; i.e., each stock would be about 1.9×10^{-7} molar. Now the experiments with the effect of copper on the rate of cleavage of Arbacia eggs indicated that copper was largely ineffective beyond an assay concentration of 10^{-13} M. This would mean that in our extracts any effective copper would have been diluted out beyond the extract concentrations of 8.4×10^{-8} mgm. extract per cc. Since Table I shows that effective acceleration of cleavage was obtained with dilutions of extract beyond this point, the active principle in the extract, it can be said fairly safely, is something other than mere copper contamination.

There is another factor which is frequently suggested as being possibly responsible for the increased rate of cleavage in dense as contrasted with sparse populations of eggs: this is the matter of a temperature differential internally imposed. Allee and Evans (1937*b*) briefly discussed this possibility and decided that it was probably not important since the dense-sparse differential is as large when populations of the same density are contrasted whether they are in 20 cu. mm. or in 220 cu. mm. of sea water, and while the difference was somewhat reduced in 2020 cu. mm., those in the denser populations still cleaved significantly faster than did the accompanying sparse populations.

If we had data from the smallest drops only, and if we made the assumption, contrary to fact, that glass conducts no heat and that no heat is lost to the air, then basing our calculations on the observations of Rogers and Cole (1925), and with the additional assumption that the specific heat of sea water plus about 5000 eggs is unity, we can account for about 30 seconds acceleration by the middle of second

cleavage. This is approximately one-half the observed difference in comparable experiments (Allee and Evans, 1937*b*). When, however, increasing the volume 11 times does not alter the time relations between dense and sparse cleavage rates, and when there is still a significant difference in 2020 cu. mm., there is no real reason for considering that the heat produced within the cleaving population is actually an important factor in speeding the cleavage in the denser populations.

The present work might be considered an extension of the field of population physiology to the field of early echinoderm development. It is, however, difficult to conceive of Arbacia development at the early stages of cleavage in terms of utilization of nutrient materials. Indeed, what we are dealing with here is essentially a unique type of population: one in which the factor of nutrition in the external environment is absent. And inasmuch as this factor of food is not involved, the environment of an experimental study of a population of this kind is more readily controlled, at least in this one respect.

From another point of view, the present work is part of a more general program of investigation, for some time under way at the University of Chicago, dealing with the phenomena of the effect of population density upon various biological processes, both in nature and in the laboratory. The demonstration of the existence of optimum densities for various processes and organisms, widely spread throughout the living world, has indicated that undercrowding is as real in its effects as is overcrowding. The present work is an extension of an attempt to gain a better understanding of the complexities of one of these situations: the population aspect of Arbacia egg cleavage. The more general implications of this work are discussed by the senior author in his "Animal Aggregations" (1931) and more recently in his 1938 book on "The Social Life of Animals."

Summary

1. Fertilized Arbacia eggs contain a readily extracted substance (or substances) which, in our tests, accelerated the rate of cleavage of such eggs to a statistically significant degree.

2. The acceleration produced by such an extract is not a result of the echinochrome it may contain nor of possible copper contamination.

3. The results are discussed against a background of population physiology, and the problems of over- and undercrowding of organisms.

LITERATURE CITED

Allee, W. C., 1931. Animal aggregations: a study in general sociology. University of Chicago Press.

Allee, W. C., 1938. The social life of animals. New York: Norton.

ALLEE, W. C., AND G. EVANS, 1937*a*. Some effects of numbers present on the rate of cleavage and early development in Arbacia. *Biol. Bull.*, **72**: 217–232.

ALLEE, W. C., AND G. EVANS, 1937*b*. Further studies of the effect of numbers on the rate of cleavage in eggs of Arbacia. *Jour. Cell. and Comp. Physiol.*, **10**: 15–28.

ALLEE, W. C., AND G. EVANS, 1937*c*. Certain effects of numbers present on the early development of the purple sea-urchin, Arbacia punctulata; a study in experimental ecology. *Ecology*, **18**: 337–345.

ALLEE, W. C., AND A. J. FINKEL, 1939. Factors affecting rate of cleavage of Arbacia: a preliminary study of homotypic extracts. (Abstract.) *Anat. Rec.*, **75**: 44 (suppl.).

ALLEE, W. C., A. J. FINKEL, AND H. R. GARNER, 1941. Factors affecting rate of cleavage in Arbacia: the accelerating action of copper. (Abstract.) *Anat. Rec.*, **81**: 125 (suppl.).

BALL, E. G., 1934. Isolation of crystalline echinochrome. *Biol. Bull.*, **67**: 327–328.

FINKEL, A. J., W. C. ALLEE, AND H. R. GARNER, 1942. Copper and the acceleration of early cleavage of Arbacia eggs. *Jour. Cell. and Comp. Physiol.*, **20**.

FRANK, G., AND M. KUREPINA, 1930. Die gegenseitige Beeinflussung der Seeigeleier als mitogenetischer Effekt betrachtet. *Arch. f. Entw. d. Org.*, **121**: 634–638.

GRAVE, C., 1935. Metamorphosis of Ascidian larvae. *Pap. Tort. Lab.*, **29**: 209–292.

HAYWOOD, C., AND W. S. ROOT, 1930. A quantitative study of the effect of carbon dioxide upon the cleavage rate of the Arbacia egg. *Biol. Bull.*, **59**: 63–70.

MAXIA, C., 1933. Ulteriori ricerche sulle radiazione "mitogenetiche." VIII. Esperimenti di mutuoinduzioni tra uova di riccio di mare. *Scritti Biol.*, **8**: 141–147.

MCCLENDON, J. F., 1912. Echinochrome, a red substance in sea urchins. *Jour. Biol. Chem.*, **11**: 435–441.

PEEBLES, F., 1929. Growth regulating substances in echinoderm larvae. *Biol. Bull.*, **57**: 176–187.

ROGERS, C. G., AND K. S. COLE, 1925. Heat production by the eggs of Arbacia punctulata during fertilization and early cleavage. *Biol. Bull.*, **49**: 338–353.

SHAPIRO, H., 1941. Centrifugal elongation of cells, and some conditions governing the return to sphericity, and cleavage time. *Jour. Cell. and Comp. Physiol.*, **18**: 61–78.

SMITH, H. W., AND G. H. A. CLOWES, 1924. The influence of carbon dioxide on the velocity of division of marine eggs. *Amer. J. Physiol.*, **68**: 183–202.

SPRINGER, M. G., 1922. The effects upon developing eggs of extracts of embryos of the same species. *Biol. Bull.*, **43**: 75–96.

"STUDENT," 1925. New tables for testing the significance of observations. *Metron*, **5** (3): 105–108, 114–120.

SUGIYAMA, M., 1938. Effect of crowding upon the rate of cleavage in sea urchin eggs. *Zool. Mag.* (Tokyo), **50**: 48–50.

A TIME STUDY OF EVENTS IN THE LIFE SPAN OF DAPHNIA MAGNA[1]

BERTIL G. ANDERSON[2] AND JOSEPH C. JENKINS

(From the Franz Theodore Stone Laboratory of the Ohio State University, Put-in-Bay, and the Biological Laboratory, Western Reserve University, Cleveland)

Cladocera have been used by many biologists as experimental animals in various types of investigations. Their use is advantageous in many respects. They are small, have a relatively short life span, and are easy to culture. They mature early and reproduce rapidly so that they are readily available in large numbers. Since reproduction can be limited to the production of females by diploid parthenogenesis, genetic constancy can be maintained. Their size and transparency allow microscopic observation of the activity of many organs and systems without resorting to operative techniques.

In spite of the fact that Cladocera have been widely used, many workers have paid little or no attention to the past history of the individual experimental animals and the stage of the life history in which they have been employed. The past history of the individual is of considerable importance in determining the results of experiments inasmuch as Ingle, Wood, and Banta (1937) have shown that well fed animals grow more rapidly, reproduce in greater numbers, and have a higher heart beat frequency than those reared on limited food. The stage in the life history is significant since heart beat frequency, oxygen consumption, and resistance to toxic materials vary with the age of the animal (Ingle, Wood, and Banta, 1937; Obreshkove, 1930; Terao, 1931; MacArthur and Baillie, 1929b; Terao and Tanaka, 1929 and 1930; and Breukelman, 1932). The time within the instar is not to be ignored since a new layer of chitin forms during the latter part of each instar (Anderson and Brown, 1930) and this may modify rates of permeability of various substances. Again their reactions may vary depending on the time within the instar although we do not know of any studies with reference to this particular point. The matter of sex should not be overlooked. Males are more susceptible to poisons than females (Breukelman, 1932). In general the metabolic activity of the males is higher than that for females (MacArthur and Baillie, 1929a). Again members of different clones show striking physiological differ-

[1] Aided by a grant from the Research Fund of the Ohio Academy of Science.

[2] Present address: Department of Botany and Zoology, West Virginia University.

ences (Obreshkove and Banta, 1930, and Banta, 1939). These considerations show that in order to secure reproducible results the individual experimental animals must be reared under adequately controlled conditions and selected with respect to age, time within the instar, sex, and clone.

Individual rearing of experimental animals offers a means whereby a complete check may be made on age, time within the instar, and their general physiological condition. For animals from mass cultures as have been used by many investigators this is only partly possible. Time studies of the events in the life span of several varieties of only one species, *Daphnia longispina*, have been made (Ingle, Wood, and Banta, 1937, and Banta, 1939). No such data are available for the most used species, *Daphnia magna*. We have, therefore, undertaken this time study of events in the life span of individually reared female *Daphnia magna* together with analyses of criteria for determining their physiological condition at the various stages in their life history.

Materials and Methods

A first consideration was culture medium. Numerous media have been employed by various investigators. One of the simplest is the manure-soil medium of Banta (1921a). We have used a modification of this with considerable success for over a decade. Air dried horse manure was used instead of fresh as stipulated by Banta. This together with soil was allowed to stand in tap water for two days after which the infusion was filtered through fine silk bolting cloth. Before using, the filtrate was permitted to stand until the turbidity disappeared, which usually occurred within two days. Medium over five days old from the time of filtering was never used.

Within an hour after their release from the brood chambers of the mothers individual females were placed in vials of 20 to 25 cubic centimeters capacity. The medium was changed at the end of the second day. On the fourth day the animals were transferred to larger vials containing 50 to 60 cubic centimeters of culture medium and every third day thereafter they were transferred to vials containing fresh medium. The smaller vials were used during the first four days merely to facilitate finding the small cast carapaces of the early instars.

Another consideration was temperature. Twenty-five degrees Centigrade was decided upon since it is easily maintained throughout most of the year with the simplest of constant temperature equipment. It is well under the lethal temperature for *Daphnia magna* (Brown, 1929) and also below the highest temperature at which normal life functions occur (MacArthur and Baillie, 1929a).

Observations were made hourly from 8 A.M. to 11 P.M. each day from the time the animals passed as eggs into the brood chamber of the mother until about five-sixths of the animals had died. Daily observations were then made until all were dead. Records were kept of the time each individual passed as an egg into the brood chamber of the mother, the time of release from the brood chamber as a free living young, the time of each ecdysis, the time of release of its young, and the number of young produced in each brood.

Number of Pre-adult Instars

One hundred fifty-nine female *Daphnia magna* Straus were observed from the time of their passage as eggs into the brood chambers of their mothers throughout their whole life span. Of these, 48 were primiparous during the fifth instar, 86 during the sixth, and 24 in the seventh, while one died in the fourth. The number of pre-adult instars, therefore, varied from four to six. This is the first time that less than five pre-adult instars have been reported for this species.[3] The variation in the number of pre-adult instars might conceivably be due to either or both hereditary and environmental factors. Inasmuch as the animals were all of one clone they were genetically identical, but mutations do arise occasionally (Banta, 1939) and this might account for the variation. However, this seems unlikely since individuals from the same brood varied. On the other hand differences in the culture medium may have been the cause. Twenty animals were set out each day over a period of eight days simply as a matter of convenience. As a consequence the animals were started out on three different batches of culture medium. We found that a particular number of pre-adult instars predominated for the animals set out on any one day. For these reasons we believe that the variation in the number of pre-adult instars was due to environmental factors.

In order to facilitate further discussion of our results the animals have been divided into three groups on the basis of the number of pre-adult instars.

Longevity

Figure 1 shows the survival curves for the animals observed. The animals primiparous in the fifth instar had a mean life span of 944 $\pm$ 32 hours ($\bar{x} \pm \sigma_{\bar{x}}$); those primiparous in the sixth, 966 $\pm$ 26 hours; and those primiparous in the seventh, 990 $\pm$ 43 hours. In terms of instars, they lived 16.5 $\pm$ 0.4; 17.2 $\pm$ 0.4; and 18.3 $\pm$ 0.6, respectively.

[3] Anderson (1932) erroneously interpreted Rammner (1930) as having found eggs produced during the fifth instar.

The differences between any two groups either in terms of hours or instars are not statistically significant. However, it is interesting to note that the average number of adult instars is the same for each group and the differences in longevity correspond to the differences in the accumulated time at the end of the pre-adult instar period.

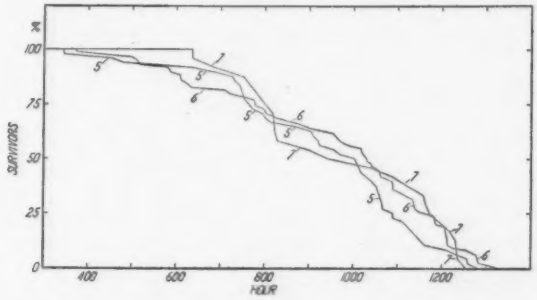

FIG. 1. Survival curves for female *Daphnia magna* primiparous during the fifth, sixth, and seventh instars as indicated by the numerals.

MacArthur and Baillie (1929a) in their experiments on the effect of temperature on longevity found that female *Daphnia magna* lived 29.24 ± 0.44 days (701.8 ± 10.6 hours) at 28° C. and 44.73 ± 0.47 days (1073.5 ± 11.3 hours) at 18° C. Our animals lived longer than would be expected from their results. In all probability our culture conditions were more favorable since, as will be brought out later, the number of young produced was greater.

DURATION OF THE INSTARS

The duration of each instar for each pre-adult instar group is given in Table I. This is also shown graphically in Figures 2, 3, and 4. In general the duration of the instars increases with age. This agrees with the results secured by Ingle, Wood, and Banta (1937) for *Daphnia longispina*. The duration of any one pre-adult instar for animals primiparous during either the fifth or sixth instars is significantly different from that of any other pre-adult instar. This does not hold true for the animals primiparous in the seventh instar. The first adult instar, the instar during which the females are primiparous is distinctly longer than the longest pre-adult instar. While the duration of adult instars varied considerably the differences are not often significant. In a few cases, notably in the eleventh and twelfth instars of the group primiparous in the fifth instar, the twelfth and thirteenth

of those primiparous in the sixth, and the thirteenth and fourteenth of those primiparous in the seventh, the instars are definitely longer than those preceding and on the average longer than those which follow. This we believe was due to a deficiency in food during those instars. This point will be discussed more fully in connection with young production in the section which follows.

TABLE I

Duration of instars in hours for animals primiparous in

Instar	Fifth Instar		Sixth Instar		Seventh Instar	
	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations
B*	49.5±0.1	48	48.9±0.2	86	47.8±0.2	24
1	20.3±0.2	48	20.4±0.2	86	20.2±0.2	24
2	21.3±0.1	43	21.3±0.2	68	24.7±0.5	18
3	24.8±0.1	43	24.1±0.2	60	37.5±1.8	19
4	32.5±0.2	34	25.5±0.1	60	24.4±0.4	15
5	50.1±0.3	16	32.5±0.4	33	23.1±0.4	17
6	52.8±1.1	5	50.8±0.4	29	30.5±0.3	21
7	57.9±0.2	13	55.0±0.5	31	48.8±0.3	23
8	59.8±0.7	22	59.3±0.8	15	52.8±0.6	15
9	58.9±1.1	18	58.1±0.5	18	54.0±2.1	3
10	62.6±1.0	19	61.0±0.8	25	55.6±1.3	8
11	70.1±1.3	14	66.1±0.8	22	57.7±0.7	13
12	68.9±1.5	11	71.3±1.3	21	62.4±0.4	11
13	66.0±0.7	11	68.0±0.8	23	76.4±1.1	14
14	66.1±0.9	12	67.9±0.9	26	70.7±2.2	10
15	66.3±1.0	6	65.8±0.9	24	64.6±0.9	9
16	67.0±0.6	15	65.2±0.5	18	69.6±0.6	7
17	70.3±0.9	17	67.0±0.8	18	65.8±1.3	5
18	72.0±1.2	9	70.2±2.0	17	66.0±0.6	5
19	67.6±0.8	5	66.2±0.9	15	66.0±0.7	2
20			74.0±0.5	6	66.3±2.9	3
21			72.5±0.6	4	66.0±2.1	2
22			82.0±7.8	2		

* Brooding period.

Here also it is interesting to compare our results with those of MacArthur and Baillie (1929a) who state that the time between broods (the equivalent of duration of the instar) was three days (72 hours) at 28° C. In very few instances did the mean durations exceed 72 hours in our observations. As we did with respect to longevity, we would interpret this to mean that our culture conditions were more satisfactory.

The accumulated time at the end of each instar is given in Table II and is graphically portrayed in Figures 2, 3, and 4. It should be noted

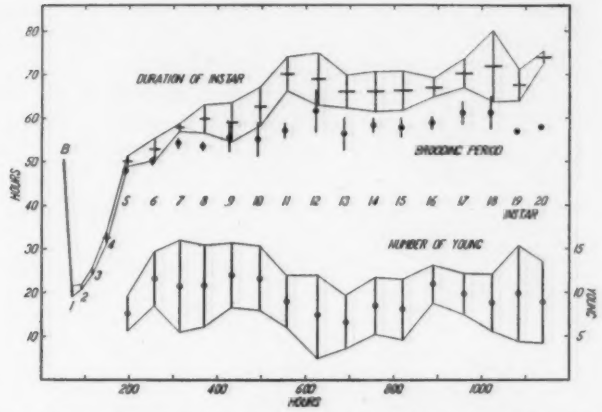

FIG. 2. Duration of individual instars, accumulated time at the end of instars, duration of the brooding periods, and the number of young released during each instar for the animals primiparous during the fifth instar. The duration of each instar is given with respect to the ordinate in hours. The average duration is the point where the bars intersect while the length of the vertical bar represents two standard deviations, one on each side of the mean. The point of intersection also represents the accumulated time with reference to the abscissa and the length of the horizontal bar represents two standard deviations from the mean. The duration of the brooding periods is in the same terms as the duration of the instars with the solid circles denoting the means. The number of young is given with respect to the right ordinate. Here also the vertical length represents two standard deviations and the open circles the means.

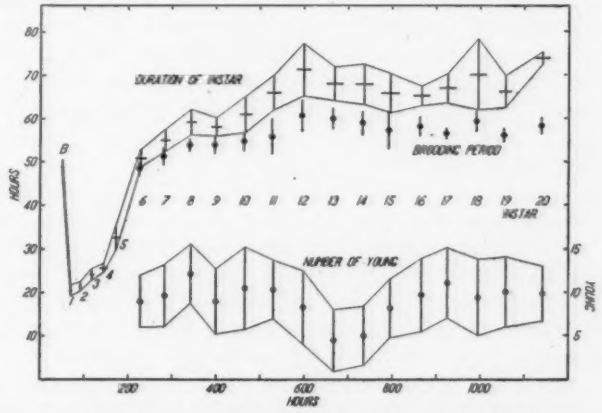

FIG. 3. Duration of instars, accumulated time at the end of instars, duration of the brooding periods, and the number of young released during each instar for the animals primiparous during the six instar. See Figure 2 for further explanation.

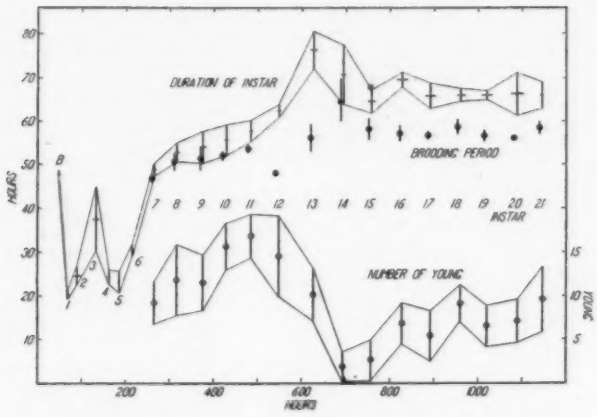

FIG. 4. Duration of instars, accumulated time at the end of instars, duration of the brooding periods, and the number of young released during each instar for the animals primiparous during the seventh instar. See Figure 2 for further explanation.

that after the third instar the accumulated time at the end of any instar for any one group was significantly different from that for the corresponding instar of any other group until the twentieth instar was reached.

REPRODUCTION

The number of young produced during each instar for each group is given in Table III and also in Figures 2, 3, and 4. In the main, the number of young produced increased for the first few adult instars after which it decreased. In two of the three groups the peak was reached in the fifth adult instar. This is similar to the course followed in *Daphnia longispina* (Ingle, Wood, and Banta, 1937; and Banta, 1939) and in *Daphnia pulex* (Anderson, Lumer, and Zupancic, 1937). Striking reductions in young production occurred during the middle of the reproductive period for each group. This we believe was due to an unsatisfactory batch of culture medium since the reduction appeared simultaneously for all the animals in the experiment. Dunham (1938) has shown that the number of young produced decreases considerably when well fed *Daphnia longispina* are placed on limited food.

MacArthur and Baillie (1929a) found that the average total young production for individual females raised at 28° C. was 15, at 18° C. 49, and at 8° C. 36. In comparison our animals produced three or more times as many. This tends to demonstrate further that our

cultural conditions were better. An alternative explanation would be that their stock was genetically different since Banta (1939) has pointed out that genetic differences often become apparent in this way.

In the preceding section we brought out the fact that certain instars were of markedly longer duration than those immediately before and

TABLE II

Accumulated time at the end of instars in hours for animals primiparous in

Instar	Fifth Instar		Sixth Instar		Seventh Instar	
	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations
B*	49.5± 0.1	48	48.9± 0.2	86	47.8±0.2	24
1	69.8± 0.3	48	69.3± 0.3	86	67.9±0.3	24
2	91.4± 0.3	43	91.7± 0.2	68	94.8±1.7	20
3	116.0± 0.3	46	116.8± 0.6	62	133.3±1.5	23
4	147.6± 0.3	35	144.2± 1.1	65	162.4±0.8	18
5	195.6± 0.4	16	171.8± 1.4	53	185.8±0.5	21
6	256.7± 1.1	19	228.4± 1.6	54	215.6±0.6	24
7	312.2± 0.5	40	283.8± 1.3	53	264.4±0.7	23
8	370.4± 1.5	30	342.0± 1.7	41	316.0±1.2	15
9	431.0± 1.6	30	398.8± 1.6	61	374.5±1.9	8
10	495.2± 1.7	36	464.7± 1.9	48	428.5±0.8	24
11	556.8± 2.7	19	530.2± 2.4	41	484.2±0.9	13
12	627.1± 2.5	22	597.4± 2.4	41	548.5±0.9	21
13	691.2± 2.2	28	668.8± 2.6	49	626.0±2.0	16
14	757.3± 3.6	20	735.7± 2.9	43	694.1±1.3	17
15	819.7± 2.7	19	795.1± 3.1	44	759.0±2.4	12
16	889.6± 1.7	22	866.2± 3.4	30	827.4±3.9	10
17	959.2± 2.3	22	924.6± 3.2	32	891.8±3.8	9
18	1025.4± 3.7	13	994.5± 3.9	29	961.6±2.8	8
19	1084.4± 7.7	5	1056.3± 3.0	21	1019.8±5.2	6
20	1178.0±19.2	2	1141.6± 4.8	11	1088.5±5.3	6
21	1206.0±	1	1206.0± 6.9	6	1145.0±2.1	2
22			1285.0±17.0	2	1249.0±	1

* Brooding period.

following. This increase in duration coincides with the decrease in number of young produced as is revealed by inspection of Figures 2, 3, and 4. Wood and Banta (1936) have pointed out that growth during any instar is negatively correlated with the duration of that instar and is directly correlated with the number of young produced in *Daphnia longispina*. Young production and the duration of an instar should, therefore, be negatively correlated. To test this point we have determined the coefficients of correlation for the number of young released during several instars and the duration of the instars

immediately preceding, during which the eggs are developed. For the animals primiparous in the fifth instar we found a coefficient of correlation of $+ .14 \pm .24$ ($r \pm \sigma_r$) for the young released in instar six and the duration of instar five; $- .68 \pm .12$ for the number of young in instar nine and the duration of instar eight; and $- .24 \pm .25$ for the young in instar 12 and the duration of the eleventh. For animals primiparous in the sixth we found $- .36 \pm .16$ for the young in the seventh instar and the duration of the sixth instar; $- .55 \pm .14$ for the young in the eleventh instar and the duration of the tenth; and

TABLE III

Number of young produced by animals primparous in

Instar	Fifth Instar		Sixth Instar		Seventh Instar	
	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations
5	7.6±0.3	48				
6	11.5±0.5	48	8.9±0.3	86		
7	10.7±0.8	48	9.6±0.4	86	9.2±0.5	24
8	10.8±0.7	47	12.1±0.4	86	11.8±0.8	24
9	11.9±0.5	47	8.9±0.4	85	11.5±0.7	24
10	11.6±0.6	46	10.5±0.5	84	15.6±0.5	24
11	9.0±0.4	45	10.3±0.4	83	16.8±0.5	24
12	7.4±0.8	45	8.3±0.5	79	14.5±0.9	24
13	6.6±0.5	44	4.5±0.4	71	10.1±0.6	24
14	8.4±0.5	40	5.0±0.4	70	1.8±0.4	22
15	8.0±0.6	32	8.1±0.4	63	2.6±0.5	22
16	10.9±0.4	32	9.7±0.6	58	6.8±0.5	20
17	9.8±0.5	28	11.0±0.5	57	5.4±0.8	14
18	8.8±0.7	21	9.4±0.7	46	9.1±0.6	12
19	9.9±1.5	13	10.0±0.6	40	6.5±0.7	12
20	8.9±1.6	8	9.8±0.6	32	7.1±0.7	11
21	3.5±0.4	2	5.7±0.7	14	9.6±1.2	10
22			5.4±1.4	5	7.8±1.8	3

$- .79 \pm .09$ for the young in instar 13 and the duration of the twelfth. For animals primiparous in the seventh instar we found $- .18 \pm .20$ for the number of young in the eigth instar and the duration of the seventh; $- .18 \pm .27$ for the number of young released in the twelfth and the duration of the eleventh; and $- .78 \pm .12$ for the number of young in the fifteenth and the duration of the fourteenth. It is thus apparent that the number of young produced and the duration of an instar are correlated negatively or not at all. Lack of correlation is to be had when there is relatively little variation in the duration of the instars (see Table I and Figures 2, 3, and 4) and whenever con-

siderable variation occurs, a high negative correlation is secured. We believe, therefore, that we are justified in concluding that the number of young produced during any instar varies inversely with the duration of that instar.

Inasmuch as the rate of reproduction is directly correlated with the growth increment (Wood and Banta, 1936; and Anderson, Lumer, and Zupancic, 1937) and negatively correlated with instar duration, the use of the rate of reproduction as an index of the general metabolic condition of a daphnid, as employed by Banta (1921b) and Anderson (1933), is more fully justified. Indeed, it is perhaps the simplest criterion that might be used since a single observation on the number of young released or on the number of eggs or embryos in the brood chamber can be made rapidly and accurately.

Duration of the Brooding Periods

We wish here to introduce the term *brooding period* to designate the time that the developing individual spends in the brood chamber of the mother, i.e. the time from the individual's passage as an egg into the brood chamber of the mother until its release as a free swimming young. Ordinarily eggs are deposited in the brood chamber within a few minutes after ecdysis and the young which develop are released shortly before the next ecdysis. In these experiments we did not check specifically whether or not eggs were deposited immediately after molting since in our experience we have found no instances of retarded egg laying. The beginning of the instar, therefore, is taken as the beginning of the brooding period. Careful observation was made to note the time of release of the young. The duration of the brooding periods is given in Table IV and in Figures 2, 3, and 4. In the main they vary with the duration of the instar. This is in contrast with the findings of Obreshkove and Fraser (1940) that the duration of the brooding periods was practically constant at 46 hours at 25° C. Whether or not the young were in the same state of development at the time of release in our experiments was not determined. It should be pointed out that the brooding period is not necessarily synonymous with embryonic period. The embryonic period is usually completed by the time the young are released. However, occasionally they may be held over in the brood chamber for some time after the embryonic period is over.

Discussion

In these experiments we have undertaken to determine the times at which various events in the life span of female *Daphnia magna* occur

when they are raised under relatively constant and reproducible conditions. The culture medium (Banta, 1921a and 1939) was completely changed at definite and frequent intervals. While this did not insure absolute constancy of environment we had what can be termed a cyclic constancy. The medium was not as satisfactory as one might wish but was no more variable than others that have been used. Algal media may have greater possibilities but even they are quite variable if Banta's (1939, p. 197) interpretation of Edlén's (1937) results are correct. Cultures made up with pure strains of bacteria are difficult

TABLE IV

Duration of the brooding period in hours for animals primiparous in

Instar	Fifth Instar		Sixth Instar		Seventh Instar	
	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations
5	48.4±0.2	19				
6	50.1±0.3	8	48.3±0.3	36		
7	54.2±0.3	17	51.3±0.3	45	46.8±0.2	23
8	53.5±0.3	15	53.9±0.3	20	50.6±0.5	20
9	55.5±0.7	21	53.9±0.4	24	51.3±1.1	6
10	55.1±1.2	10	54.7±0.4	24	52.0±0.4	7
11	57.1±0.4	16	55.9±1.0	17	53.5±0.2	21
12	61.6±1.8	7	60.6±0.9	15	48.0±	1
13	56.4±1.3	8	60.0±0.6	15	56.1±1.1	8
14	58.3±0.5	9	59.0±0.9	8	64.5±3.9	2
15	57.8±0.9	6	57.3±1.2	14	58.2±1.1	5
16	58.9±0.5	10	58.2±0.6	17	57.2±0.7	6
17	61.1±0.9	7	56.5±0.4	8	57.7±0.3	3
18	61.2±1.7	5	59.4±0.6	14	58.7±1.0	3
19	57.0±	1	56.1±0.6	7	56.7±0.7	3
20	58.0±	1	58.4±0.6	12	56.0±	1
21			60.7±2.6	3	52.0±5.0	3
22			64.3±2.6	3	58.5±1.1	2

to manipulate and not entirely satisfactory in other respects (Banta, personal communication). We have tried various culture media with yeast as a basis but they have been found wanting in many ways. Of the various culture media that have been used successfully for mass culturing (Galtsoff, Lutz, Welch, and Needham, 1937) few lend themselves for the individual culturing of Daphnia. A medium is needed that can be controlled and reproduced not only by an individual investigator but by all with a minimum of effort. Until such a medium is developed the variations in the responses of Daphnia under many types of experimental conditions will be great.

Summary

Hourly observations were made on 159 *Daphnia magna* individually reared in manure-soil medium at 25° C. throughout their whole life span.

The number of pre-adult instars varied from four to six, the most frequent number being five.

The average longevity was approximately 960 hours or 17 instars.

In general the duration of the instars increases with age. The pre-adult instars with the exception of the last require about a day, the final taking about 30 to 32 hours. Beginning with the first adult the instars last approximately two days and increase slightly with each instar.

The number of young produced increases with each adult instar up to the fifth, followed by a decrease. The use of the number of young produced as an index of the general metabolic condition of a daphnid is analysed and believed justified.

The brooding periods, i.e. the time between the deposition of eggs and their subsequent release as young, vary with the duration of the instars.

The need for a more readily controllable and reproducible culture medium is pointed out.

LITERATURE CITED

ANDERSON, B. G., 1932. The number of pre-adult instars, growth, relative growth, and variation in Daphnia magna. *Biol. Bull.*, **63**: 81–98.

ANDERSON, B. G., 1933. Regeneration in the carapace of Daphnia magna. I. The relation between the amount of regeneration and the area of the wound during single adult instars. *Biol. Bull.*, **64**: 70–85.

ANDERSON, B. G. AND L. A. BROWN, 1930. A study of chitin secretion in Daphnia magna. *Physiol. Zoöl.*, **3**: 485–493.

ANDERSON, B. G., H. LUMER, AND L. J. ZUPANCIC, JR., 1937. Growth and variability in Daphnia pulex. *Biol. Bull.*, **73**: 444–463.

BANTA, A. M., 1921a. A convenient culture medium for daphnids. *Science*, **53**: 557–558.

BANTA, A. M., 1921b. Selection in Cladocera on the basis of a physiological character. *Carnegie Institution of Washington, Publication No.* 305.

BANTA, A. M., 1939. Studies on the physiology, genetics, and evolution of some Cladocera. *Carnegie Institution of Washington, Publication No.* 513.

BREUKELMAN, J., 1932. Effect of age and sex on resistance of daphnids to mercuric chloride. *Science*, **76**: 302.

BROWN, L. A., 1929. The natural history of cladocerans in relation to temperature. I. Distribution and the temperature limits for vital activities. *Amer. Nat.*, **63**: 248–264.

DUNHAM, H. H., 1938. Abundant feeding followed by restricted feeding and longevity in Daphnia. *Physiol. Zoöl.*, **11**: 399–407.

EDLÉN, Å., 1937. Experimentelle Wachstumsstudien an Daphnia magna. *Lunds Universitets Årsskrift. N. F. Avd.* 2, **24**: 1–29.

GALTSOFF, P. S., F. E. LUTZ, P. S. WELCH, AND J. G. NEEDHAM, 1937. Culture methods for invertebrate animals. Ithaca, N. Y., Comstock Publishing Co.

INGLE, L., T. R. WOOD, AND A. M. BANTA, 1937. A study of longevity, growth, reproduction, and heart rate in Daphnia longispina as influenced by limitations in quantity of food. *Jour. Exper. Zoöl.*, **76**: 325–352.

MACARTHUR, J. W. AND W. H. T. BAILLIE, 1929a. Metabolic activity and duration of life. I. Influence of temperature on longevity in Daphnia magna. *Jour. Exper. Zoöl.*, **53**: 221–242.

MACARTHUR, J. W. AND W. H. T. BAILLIE, 1929b. Metabolic activity and duration of life. II. Metabolic rates and their relation to longevity in Daphnia magna. *Jour. Exper. Zoöl.*, **53**: 243–268.

OBRESHKOVE, V., 1930. Oxygen consumption in the developmental stages of a cladoceran. *Physiol. Zoöl.*, **3**: 271–282.

OBRESHKOVE, V. AND A. M. BANTA, 1930. A study of the rate of oxygen consumption of different Cladocera clones derived originally from a single mother. *Physiol. Zoöl.*, **3**: 1–8.

OBRESHKOVE, V. AND A. W. FRASER, 1940. Growth and differentiation of Daphnia magna eggs in vitro. *Biol. Bull.*, **78**: 428–436.

RAMMNER, W., 1930. Über milieubedingte Missbildungen bei Daphnia pulex und Daphnia magna. *Int. Rev. Hydrob. Hydrogr.*, **24**: 1–23.

TERAO, A., 1931. Change of vitality with age as based on the living unit of organisms. I. Oxygen consumption in the daphnid, Simocephalus exspinosus. *Proc. Imp. Acad.*, **7**: 23–25.

TERAO, A. AND T. TANAKA, 1929. Anaesthesia of the water-flea, Moina macrocopa Straus, in relation to age. *Jour. Imp. Fisheries Inst.*, **25**: 13–14.

TERAO, A. AND T. TANAKA, 1930. Exponential decrease of susceptibility to anaesthesia with age in the water-flea, Moina macrocopa Straus. *Proc. Imp. Acad.*, **6**: 208–211.

WOOD, T. R. AND A. M. BANTA, 1936. Interpretations of data on growth and reproduction (Daphnia longispina). *Anat. Rec.*, **67**: 55.

DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER

V. Factors Influencing the Length of the Cycle; Observations upon the Gaseous and Dissolved Organic Nitrogen [1]

THEODOR VON BRAND, NORRIS W. RAKESTRAW AND J. WILLIAM ZABOR

(From the Woods Hole Oceanographic Institution, Woods Hole)

The experiments to be described in this paper are in part a continuation and amplification of studies previously reported (1937–1941), on the influence of temperature, the nature of the organic matter and the source of the water upon the speed of the nitrogen cycle, as well as the interrelation of the various steps involved. Our previous studies dealt almost exclusively with the fate of the particulate, ammonia, nitrite and nitrate nitrogen; in the present investigation we have also studied the behavior of two other important nitrogen fractions commonly found in sea water, dissolved gaseous nitrogen and dissolved organic nitrogen. Because of technical difficulties our study of these two nitrogen fractions is incomplete and merely preliminary. Nevertheless, the results are included in the present report, since it appears doubtful that the investigation can be continued in the near future.

The general method of the present experiments was the same as that previously described. Particulate organic matter, usually in the form of diatoms (*Nitzschia Closterium*), was added to samples of sea water in large carboys, which were kept in the dark while decomposition proceeded. Samples were removed from time to time for analysis of the various forms of nitrogen, generally particulate nitrogen, ammonia, nitrite and nitrate.

Influence of Low Temperature

In September, 1938, a culture (No. 28) of harbor water and Nitzschia was stored in the dark at 1° to 2° C. As reported previously (von Brand and Rakestraw, 1940), ammonia was the only decomposition product which had appeared up to June, 1940. A continued observation of this culture to June 19, 1941, failed to reveal any further change. It was transferred on this day to room temperature, but still kept dark. On June 27 nitrite had begun to appear, reaching its maximum ten days

[1] Contribution No. 321 from the Woods Hole Oceanographic Institution.

later and declining throughout August. The cycle was ended at the beginning of September, the nitrite having been quantitatively transformed into nitrate. A part of this culture was separated on June 27 and kept in a refrigerator at 5° C. The nitrite and ammonia in this sample remained constant to the end of September, when its observation was discontinued. Apparently the organisms responsible for nitrite formation did not develop at low temperatures. The rapid appearance of nitrite upon restoration to higher temperature, in the main portion of No. 28, proves that they were not killed by the low temperature, but only kept inactive.

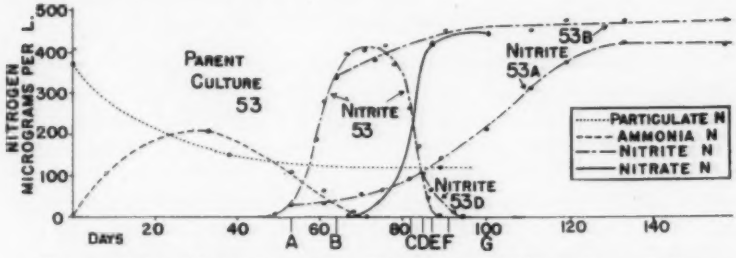

FIG. 1. Effect of low temperature. Time in days. Different forms of nitrogen in micrograms per liter. The parent culture, No. 53, consisted of Woods Hole harbor water with washed diatoms (*Nitzschia Closterium*) added. Decomposition in the dark at 20° C. Sub-cultures were removed at points on the time-scale marked *A*, *B* and *D* and preserved at 5° C. for observation of nitrite and nitrate formation, which proceeded more slowly in 53*A*, 53*B* and 53*D* than in 53. Points *C*, *E*, *F* and *G* indicate times at which other sub-cultures were removed, which are shown in Figure 5.

Series 28 is not typical of all cultures. Figure 1 shows the results in another series, Number 53. This culture also contained Nitzschia in sea water and was kept in the dark at room temperature. Sub-cultures were separated from the parent culture at various times during the decomposition, marked *A*, *B* and *D* in the figure. These were placed in a refrigerator at 5° C. and analyzed periodically, along with the parent culture, which was kept at room temperature.

The first of these sub-cultures, No. 53*A*, was separated when nitrite had just begun to appear in No. 53. Nitrite formation continued in 53*A*, but very much more slowly. At room temperature (about 20° C.) the nitrite maximum was reached 13 days after separation of the two cultures; at 5° C. only after 67 days. The average daily nitrite production was in the former case 28 micrograms per liter, the latter 5.8. The overall Q_{10} was around 3, a normal coefficient for a biological process.

Sub-culture 53*B* was separated from 53 when the nitrite was near its maximum. Nitrite continued to increase slowly in the cold, even

to a slightly higher level than in the original culture, but up to the end of the observation no nitrate had been formed.

The striking difference in temperature coefficients of nitrite formation in Series 28 and 53 might indicate that different organisms were involved. It should be kept in mind that in nature nitrite is formed at all latitudes, in waters of different temperatures. An investigation of the temperature coefficients of nitrite formation in waters of different localities might yield interesting results.

Sub-culture 53*D* was separated from No. 53 when nitrate formation in the latter was well under way. All the remaining nitrite was oxidized to nitrate in Series 53 in four days, whereas the process required nine days at the low temperature. During these periods the average daily nitrate formation was 26 micrograms per liter at the higher temperature and 11.6 at the lower. The overall Q_{10} for nitrate formation was therefore about 1.5, distinctly less than the coefficient for nitrite formation in the same series.

Nature of Particulate Matter and Source of Water

Previous studies have shown that the speed with which the nitrogen cycle proceeds is largely determined by the nature of the particulate

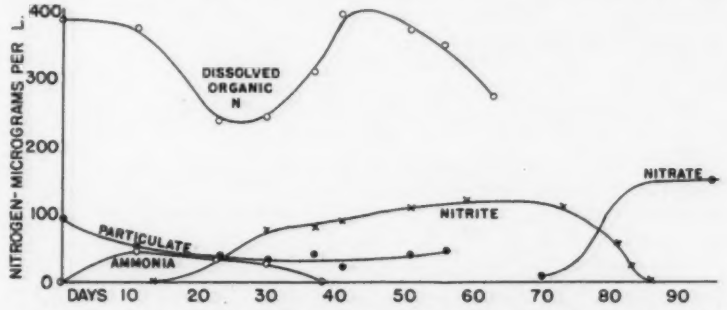

Fig. 2. Series 54. Woods Hole harbor water, with washed diatoms (*Nitzschia Closterium*). Decomposition in the dark at room temperature. Time in days. Different forms of nitrogen in micrograms per liter.

matter. The most rapid rate of decomposition was found with mixed plankton, a slower rate with diatoms, and a still slower one with plant protoplasm foreign to sea water (yeast cells). To study this relation further we undertook to compare the decomposition of diatoms (Nitzschia) with that of a protozoan (*Tetrahymena gelei*) grown in bacteria-free culture. For this purpose, two decomposition series of Nitzschia were started, No. 54 (Figure 2) in harbor water, and No. 55 (Figure 3) in deep-sea water collected from below 1000 meters depth.

In Series 57, containing harbor water, and in Series 58, containing deep-sea water, quantities of the ciliate, Tetrahymena, were introduced, which had been separated by centrifugation from the proteose-peptone culture medium and repeatedly washed with nitrate-free sea water. During this process the ciliates died but did not seem to disintegrate into too small particles to be recovered by the usual precipitation procedure after suspension.

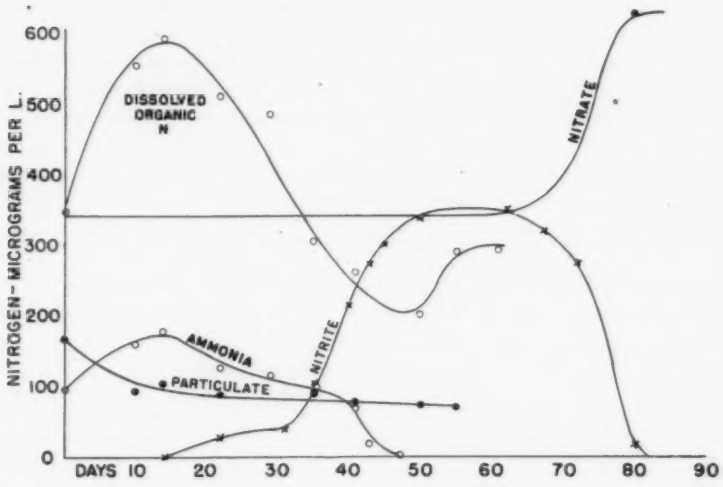

FIG. 3. Series 55. Deep-sea water (from below 1000 meters depth), with washed diatoms (*Nitzschia Closterium*). Decomposition in the dark at room temperature. Time in days. Different forms of nitrogen in micrograms per liter.

The results of Series 57 and 58 are shown in Figure 4. In both these the concentration of particulate matter was considerably higher than in Nos. 54 and 55, but it has been shown previously that this does not alter the speed of the cycle to any considerable extent.

Contrary to our expectations, the decomposition of the diatoms proceeded equally well in the harbor water and the deep-sea water. The rate of decomposition of the ciliates in harbor water was about the same as that of the diatoms. In deep-sea water, however, the decomposition of ciliates formed ammonia rapidly, but the production of nitrite was considerably retarded, and no nitrate had appeared by the time the experiment was discontinued for lack of material. The reason for this behavior is not clear.

Interrelationship of Various Steps

In our last report (1941) we pointed out that if new organic matter is added to a decomposing culture the normal sequence of "step by step" decomposition is disturbed and that under certain conditions ammonia, nitrite and nitrate formation seem to proceed at the same time. Since this situation is probably very common in nature it seemed desirable to extend the experimental study of it. Consequently, several sub-cultures were separated from Series 53, at various stages of the cycle, and new particulate matter in the form of Nitzschia was added to each. The original parent culture and the sub-cultures

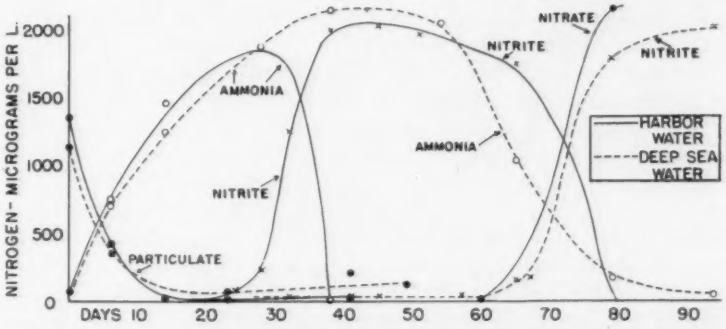

FIG. 4. Series 57 and 58. Washed cultures of *Tetrahymena gelei* suspended in Woods Hole harbor water (No. 57) and in deep-sea water (No. 58, below 1000 meters depth). Time in days. Different forms of nitrogen in micrograms per liter.

were kept in the dark at room temperature. Figure 1 shows the points at which separations were made (*C*, *E*, *F*, *G*) and Figure 5 shows the subsequent course in these sub-cultures. Series 53*C* was separated as the nitrite began to decline, 53*E* when nearly all the nitrite in Series 53 had been oxidized to nitrate, 53*F* about a week after the cycle in 53 had been completed, and 53*G* another week later.

In 53*C* practically no ammonia appeared, and the nitrite disappeared at about the same rate as in the parent culture. But due to the addition of new particulate matter the nitrate level reached a considerably higher maximum than in the parent culture. The only possible explanation is that all three processes went on at the same time, and since nitrate formation is the most rapid step, neither ammonia nor nitrite could accumulate in large amounts. This explanation is further supported by the results of sub-cultures 53*E*, *F* and *G*, separated at later stages in the cycle of the parent culture, after all the nitrogen had been completely oxidized to nitrate. In each of these ammonia

and nitrite appeared, but only in small amounts in the presence of rapid nitrate formation. This would seem to dispose of one possibility that has been suggested: that a vigorous nitrate-forming flora is capable of acting on a substratum of nitrogen compounds of high molecular weight without the formation of ammonia or nitrite as intermediates.

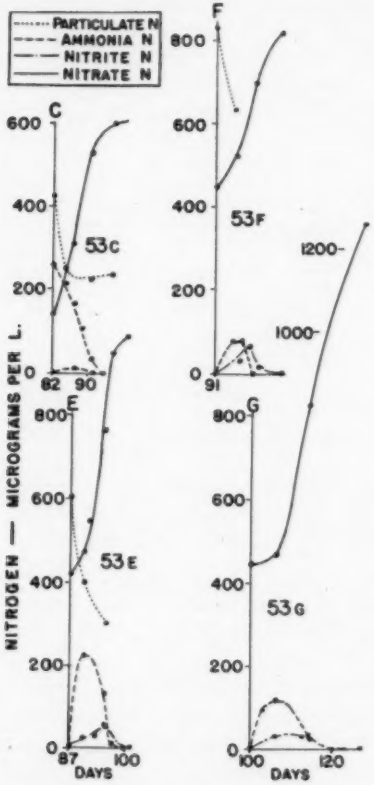

FIG. 5. Interrelationship of various steps. From parent culture No. 53, shown in Figure 1, sub-cultures were removed at times indicated by points *C*, *E*, *F* and *G*. Sub-culture 53*C* was separated when nitrate formation in 53 was proceeding rapidly; 53*E*, when nitrate formation was nearly complete; 53*F* and 53*G*, after the cycle had been entirely completed in 53. To these, new quantities of particulate organic mattre (*Nitzschia Closterium*) were added, the initial amounts being shown by the beginning of the dotted lines (except in 53*G*, in which no particulate-N analysis was made). These were preserved in the dark at 20° C. with the parent culture, No. 53. Time, in days, is measured from the beginning of culture No. 53. Different forms of nitrogen in micrograms per liter.

Another point that is again brought out in this series of sub-cultures is the acceleration of the cycle by a recurrent supply of particulate matter. The parent culture required 89 days for the completion of the cycle, the sub-cultures on the average only 18 days. The difference between the parent culture and the sub-cultures in this case is greater than in that described in our last report (1941). This is probably due to the fact that in the present case the particulate matter was added at a later stage in the decomposition cycle, when the most effective flora—the nitrate-forming one—was very vigorous. A continuation of these observations may lead to an understanding of the rapid succession of different organisms so frequently observed in the sea.

One further point should be noted. We had found previously that it was possible to repeat the "step by step" decomposition cycle in the same water at least three times, if the nitrate was regenerated into particulate matter between cycles by inoculating the culture with a few diatoms and exposing it to light for a period of about ten days. Why then did Series 53*G*, which was supplied with new organic matter two weeks after the original cycle had ended, not yield a "step by step" decomposition but rather a "simultaneous" one? We have no definite answer to this question, but it would appear that when the new organic matter is generated during a period of exposure to light the action of nitrite and nitrate forming organisms is somehow retarded, but when organic matter is introduced from an outside source there is no such inhibiting effect.

Gaseous Nitrogen

We have already pointed out that the quantitative nitrogen balance was satisfactory in about half of our experiments, while in the other half a more or less steady increase in total nitrogen occurred, for which no entirely satisfactory explanation has been found. One of the possibilities discussed was that of the fixation of elementary nitrogen by micro-organisms. Series 56 and 60 were designed to test this possibility. The technical difficulty involved was considerable and it was not possible to secure data for the whole decomposition cycle. It was necessary to use sealed samples in order to eliminate any exchange of gaseous nitrogen between the sample and the atmosphere. During the course of the decomposition, however, a large amount of oxygen is used, much more, as a matter of fact, than is found dissolved in a given sample of sea water. It was therefore necessary to introduce extra oxygen into the sample before sealing.

A procedure was devised by which a measured sample of water, containing particulate organic matter, could be introduced into a flask of about 500 ml. capacity, the small remaining air-space filled with pure

oxygen and the flask sealed off without any further contact of the sample with the atmosphere.

Nitrogen of course distributes itself immediately between the liquid and gas phases, but when analysis is carried out later the gas bubble is first removed, its volume measured and its nitrogen content determined. Since the total volume of the water sample is known an appropriate allowance can be made for the nitrogen in the gas phase after analysis of duplicate water samples by the method of Rakestraw and Emmel (1937).

The water not required for these analyses was later used for the determination of the other nitrogen fractions, as indicated in Tables I and II. The variations in gaseous nitrogen observed were small and

TABLE I

Series 56. Woods Hole harbor water, with fresh culture Nitzschia Closterium. Samples sealed in 500 ml. flasks with excess oxygen. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate	Dissolved organic	Gaseous
6–30–41	333	80	0	10	301	11770
"	316				333	
7–14	83	152	0		452	
7–28	78	225	0	15	443	
8– 4	70	330	0	15	270	11880
8–15	59	0	285*	65	430	11980
8–21	129	420	0	25	272	11990

* Since each sample analyzed is in a separate flask and decomposition may go more rapidly in some than in others, such irregularity is not surprising.

TABLE II

Series 60. Woods Hole harbor water, with fresh culture Nitzschia Closterium. Samples sealed in 500 ml. flasks with excess oxygen. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate	Dissolved organic	Gaseous
8– 7–41	141	25	0	15	336	11800
8–14	39	205	0	10	223	11760
8–20	35	175	0	20	319	11910
8–25	38	152	0	15	320	12000

probably within the limits of error of the method. But, while nitrogen fixation has not been demonstrated by these experiments, it should be pointed out that they cover only the first part of the nitrogen cycle and that at least in Series 60 no increase in total nitrogen was observed. Experiments are now under way to determine whether longer periods of decomposition are accompanied by changes in gaseous nitrogen.

Dissolved Organic Nitrogen

Another source of nitrogen that might conceivably account for the increase in total nitrogen is the dissolved organic fraction. Its behavior was studied in Series 54, 55, 56 and 60. The method used was that of Krogh and Keys (1934) with the slight modifications introduced by von Brand and Rakestraw (1941).

The results were not entirely conclusive. Easiest to understand are Series 55 (Figure 3) and 56 (Table I), where concurrently with the drop in particulate nitrogen an increase in dissolved organic nitrogen could be observed. This can be explained on the assumption that soluble nitrogen compounds were formed, intermediate between particulate nitrogen and ammonia. That a similar rise was not observed in Series 54 (Figure 2) and 60 (Table II) may be due to the more rapid formation of ammonia. In Series 54, however, a rather considerable drop in dissolved organic nitrogen occurred after the ammonia had reached its maximum. It seems to lie outside the limits of error of the method, but we cannot account for it. It should be remembered that the method was worked out for natural sea water, and it is quite possible that during the course of decomposition of a relatively large amount of particulate matter substances may be formed which interfere, a possibility which is unfortunately difficult to test.

The general impression gained from these preliminary experiments is that the dissolved organic nitrogen may be involved to some extent in the nitrogen cycle. The changes which have thus far been observed, however, are not sufficient to explain the nitrogen increase in some of our experiments. The most likely explanation for this is still contamination from outside sources, which is difficult to avoid in view of the very small quantities of nitrogen involved.

Summary

1. Low temperature may inhibit nitrite formation completely or may only retard it, probably depending upon the particular organisms present.

2. Oxidation of nitrite to nitrate was less inhibited by low temperature than the formation of nitrite from ammonia.

3. No clear-cut difference was observed between the decomposition of diatoms and that of ciliates.

4. Clear evidence was found that under certain conditions ammonia, nitrite and nitrate formation can go on simultaneously.

5. Preliminary experiments gave no evidence for a fixation of nitrogen, but indicated that dissolved organic nitrogen may possibly be involved in the nitrogen cycle.

BIBLIOGRAPHY

KROGH, A., AND A. KEYS, 1934. Methods for the determination of dissolved organic carbon and nitrogen in sea water. *Biol. Bull.*, **67**: 132–144.

RAKESTRAW, N. W., AND V. M. EMMEL, 1937. The determination of dissolved nitrogen in water. *Ind. and Eng. Chem.* (Anal. Ed.), **9**: 344–346.

VON BRAND, T. AND N. W. RAKESTRAW, 1940. Decomposition and regeneration of nitrogenous organic matter in sea water. III. Influence of temperature and source and condition of water. *Biol. Bull.*, **79**: 231–236.

VON BRAND, T. AND N. W. RAKESTRAW, 1941. Decomposition and regeneration of nitrogenous organic matter in sea water. IV. Interrelationship of various stages; influence of concentration and nature of particulate matter. *Biol. Bull.*, **81**: 63–69.

VON BRAND, T. AND N. W. RAKESTRAW, 1941. The determination of dissolved organic nitrogen in sea water. *Jour. Mar. Res.*, **4**: 76–80.

VON BRAND, T., N. W. RAKESTRAW AND C. E. RENN, 1937. The experimental decomposition and regeneration of nitrogenous organic matter in sea water. *Biol. Bull.*, **72**: 165–175.

VON BRAND, T., N. W. RAKESTRAW AND C. E. RENN, 1939. Further experiments on the decomposition and regeneration of nitrogenous organic matter in sea water. *Biol. Bull.*, **77**: 285–296.

THE EFFECTS OF POPULATION DENSITY UPON GROWTH AND SIZE IN LYMNAEA PALUSTRIS

GRACE SPRINGER FORBES AND HENRY E. CRAMPTON

(From the Department of Zoology, Barnard College, Columbia University, New York)

INTRODUCTION

There is a voluminous literature on the effects of increasing or of decreasing population density. Much of the work has been done on animal aggregations confined in a small space rather than on groups of animals held together by social instinct. In some cases beneficial effects have been reported as the result of a moderate degree of crowding, and it has been suggested that such primitive advantages of association may have played a considerable part in the development of social organization. Among the lower forms such as protozoa, worms, crustacea, fish and amphibia, animals in an aggregation may be better able to withstand the effects of toxic substances in the water medium, or of extremes of temperature, than when they are living in isolation. It has been found that under certain circumstances associated animals condition the water more favorably, perhaps by adding some important substance to it, with the result that fertility or the rate of growth may be increased. In Drosophila longevity is increased to a certain degree by an increase in the density of the population, and in Tribolium the reproductive rate shows a similar relationship to population density. Summaries and critical reviews of this subject are given by Allee (1930) and by Hammond (1938, 1939).

The workers on aquatic snails have given conflicting interpretations of their tests of the effects of population density upon rate of growth, although most of them agree that even moderate crowding brings about a reduction in size. Hoffman (1927) has given a summary of the earlier literature bearing on this subject, and Crabb (1929) showed that marked retardation of growth in the presence of adequate food in *Lymnaea stagnalis appressa* may result from crowding. However, the data of some of the researches have been too scanty to be conclusive, while in many of them the natural range of variation has not been taken into account.

We have carried on extensive laboratory experiments on *Lymnaea palustris* for several years and have reared seven consecutive generations. The original animals came from a mill-pond in Newtown,

Connecticut. Scores of families and thousands of individuals have been obtained, and abundant data for numerous biological problems have been accumulated. The present paper records some of our observations on the effects of crowding upon growth and size.

Methods

In our standardized procedure, the young snails that developed from a single mass of eggs were kept together in a large nursery jar, in about two liters of water, until they were a specified age, when they were measured and isolated in quart jars containing from 500 cc. to 600 cc. of fresh spring water. Each jar contained a spray of Cabomba and a little finely-sifted earth. Green loose-leaf lettuce was used as food, and a surplus was always kept in the jars. The snails were housed in a greenhouse during the winter months until the temperature became too warm, toward the end of May, when they were brought down to the cooler laboratories.

Efforts were made to maintain a temperature as close to 70° F. as possible. The parent snails were kept in isolation, hence all the eggs deposited were self-fertilized (Crabb, 1927).

The jars were kept clear and free from scum and obvious bacterial contamination, although by no means sterile. The water level was kept constant by adding fresh water whenever needed, at least once a week. In the tripartite experiments fresh water was used for all the groups, and in no case was old medium returned to the jars.

The length of the shell was taken as the index of size. The F test was employed to determine the significance of a difference between two comparable groups. In our tables, N is the number of items in the sample or group, $\bar{X}$ is the arithmetical mean, while r is the usual coefficient of correlation. F is a critical value which, taken in conjunction with the proper degrees of freedom, may be denoted as significant or not significant by reference to an F table such as that available in "Applied General Statistics" by Croxton and Cowden (pp. 878–879). The degrees of freedom involved are: between the means, $n_1 = 1$; and within the samples, $n_2 = N_1 - 1 + N_2 - 1$, where N_1 is the number of individuals in one sample and N_2 is the number of individuals in the other sample. The level of significance adopted was .05.

Experimental Results

The fundamental problem is whether there is a correlation between the number of snails in a family aggregation and the average size of the

members of such a group. For this problem we have available a series of 54 families with varying numbers, comprising 1056 individuals in all. The animals of each family were reared together until the age of 80 days, when they were measured. The results of the statistical

TABLE I

Data of size at successive age periods

Family	80 days			160 days		
	N	$\bar{X}$	S	N	$\bar{X}$	S
		mm.			mm.	
A	30	4.4630	1.5101	28	6.5714	1.7686
B	37	5.3486	2.1224	22	7.5000	2.1012
C	41	2.8415	0.8628	30	5.1300	1.1847
D	49	4.6143	2.0363	46	7.7152	2.8495
E	41	6.0927	1.3378	34	6.3706	1.2440
F	44	4.7591	0.9641	43	6.4302	1.7660
G	33	2.6636	0.8605	23	8.1739	1.8811

Family	240 days			320 days		
	N	$\bar{X}$	S	N	$\bar{X}$	S
		mm.			mm.	
A upper	6	17.7333	1.7489	3	20.5333	0.4619
middle	16	8.1500	0.9388	12	11.0500	1.4526
lower	6	19.3500	1.2486	2	21.9000	0.8485
B upper	6	15.1667	2.6905	2	17.6500	4.3134
middle	4	11.4000	2.3036	0	——	——
lower	4	10.6750	4.0401	1	11.2000	——
C upper	4	16.6750	1.2230	2	22.4000	0.1414
middle	11	7.9091	0.7674	7	11.1000	0.8145
lower	5	15.4200	1.8566	4	18.8750	1.4773
D upper	6	18.4333	0.9331	4	19.1250	0.2986
middle	30	8.8567	2.2773	0	——	——
lower	5	16.7400	1.2798	4	17.1250	1.1701
E upper	5	16.4600	2.2052	3	18.1000	1.9000
middle	14	8.5714	1.6452	2	10.6500	2.0506
lower	3	14.6333	2.0257	3	16.1666	1.5308
F upper	6	20.2333	2.2757	2	21.3500	1.4849
middle	2	9.9500	0.0707	2	16.5500	2.4749
lower	4	20.6250	1.8392	1	19.0000	——
G upper	6	21.2667	1.6170	1	21.5000	——
middle	1	14.8000	——	—	——	——
lower	6	20.0333	0.6088	6	21.2500	0.7868

treatment were as follows:

N	SX	SX^2	SY	SY^2	SXY	F	Significance
54	1056	28884	374.26	2947.60	6484.10	6.987	positive

The figures definitely prove the reality of a highly ***significant negative correlation*** between the number in the nursery and the average size of the individuals composing the family group. Hence the effect of an increase in the density of the population is to reduce the size of the snails.

Two families composed of approximately the same numbers of individuals were procured from the same parent. The members of the first were isolated at hatching when they were 18 days old, while the members of the second were kept together until the age of 80 days, when they also were isolated. All were measured again at the age of 160 days. The results of their statistical comparison are as follows:

Difference in size at 80 days

	N	$\bar{X}$ mm.	SX^2	F	Significance
Family I	16	14.0625			
Family II	14	8.7071			
			4277.47	115.2926	positive

Difference in size at 160 days

Family I	16	15.7562			
Family II	14	14.3742	6909.71	8.711	none

The figures show that the effect of moderate crowding between hatching and the age of 80 days is real. In the case of Family II, they also show that escape from the effects of crowding occurred after the animals were isolated, and that this escape was practically complete at 160 days.

By random sampling, two additional groups were assembled from the available general population. As in the foregoing case, the first group comprised individuals which had been isolated at hatching while the members of the second group had grown together in their respective family aggregations until they were 80 days old. All were measured at 80 days of age. The comparison of the two groups gave the following results:

	N	$\bar{X}$ mm.	SX^2	F	Significance
Group I	26	13.6153			
Group II	35	5.8885			
			4858.28	235.694	positive

The lesser average size ($\bar{X}$) of the members of Group II shows that here

too the effect of population density is to reduce the amount of growth of the animals in an aggregation. The greater value of F in this instance, as compared with that in the preceding analysis, may be due to greater inequalities in the numbers of the comparable groups, or to genetic divergence and distinction.

We now present additional data that not only confirm the conclusions based on the previous material but also show to what extent recovery from the effects of crowding may ensue upon isolation. The subjoined data relating to three different families give the average sizes, in millimeters, of the members of these families at successive times in their lives, as follows:

	80 days		160 days		240 days		320 days		400 days	
	N	Size	*N*	Size	*N*	Size	*N*	Size	*N*	Size
		mm.		*mm.*		*mm.*		*mm.*		*mm.*
Family I	39	4.24	39	14.34	39	17.22	19	19.98	—	—
Family II	17	6.56	16	7.37	13	14.88	7	18.08	5	17.90
Family III	48	3.56	48	6.39	11	11.31	10	14.00	6	16.17

In the experiments in question, the individuals of Family I were isolated at 80 days, when the average size was only 4.24 mm. Their subsequent growth to more than 14 mm. at 160 days was about as usual under the circumstances. Family II was allowed to grow as an aggregation until 160 days, when its members were isolated. The average size very nearly equalled that of the first group at the 320-day period. The snails of Family III were not separated until they were 320 days of age at which time the ten surviving individuals had gained the average size of only 14 mm., a figure considerably below the average sizes of the other two groups at the same period. The three families had the same genetic origin as they were all grown from eggs laid by the same parent.

The data show that the longer the snails were kept in an aggregation, the less able they were to recover from the inhibiting effects of crowding. Nevertheless, crowding during only the first 80-day period had little, if any, effect upon final size. The normal rate of growth is evidently susceptible to modification during the earlier stages without permanent result, but if the effects of crowding are prolonged, full recovery does not follow.

Still another procedure was followed in the present series of studies. A single family was reared in a nursery jar; the animals were measured at 80 days and were then returned to the same jar where they remained

until they were 160 days old. At this time they were measured again, and the six largest and six smallest individuals were isolated, while the intermediate members of the graduated series were kept together in the original nursery. Not a single individual died before the age of 240 days when all were again measured, but some members of each of the three subordinate groups failed to reach the age of 320 days. The average sizes of the members of the groups are given for the several age-intervals as follows:

	80 days	160 days	240 days	320 days
	mm.	mm.	mm.	mm.
Upper 6	7.00	9.50	17.83	20.50
Middle 16	4.28	6.37	8.26	11.25
Lower 6	2.83	5.17	19.17	22.00

The six largest snails, constituting the "upper" group at 160 days had evidently grown at a more rapid rate than the others, either by virtue of an inherent predisposition to do so, or because they were relatively less susceptible to the inhibiting effects of crowding. On the assumption that all of the snails had kept their places in the scale of size between 80 and 160 days, it would seem that the absolute amounts of growth were about the same for the three series, although the relative amounts were different. Considering the figures for 240 days, it is clear that both the upper and the lower groups grew very rapidly after the time of isolation, while the components of the middle group were markedly retarded as the result of their continued aggregation. At 320 days the 12 surviving snails of the intermediate group averaged only 11.25 mm. in length, a figure that is only a little more than half of the average for the lower group. The two living members of the last-named were actually longer than the survivors of the upper series at this last age interval; but their relation was exceptional.

Seven additional families of the third pedigreed generation belonging to a single clone were treated in the same way to ascertain whether the above relations and phenomena were consistent. The results are given in Table I. The several families differed somewhat in the quality of average size at 80 days, but the figures are uniformly low. In all of them the amount of growth from 80 days to 160 days was relatively small because the animals were living in association. In every case the components of the upper and lower groups enlarged at a notably rapid rate after their isolation. Also in every case the animals of the intermediate groups which were not isolated lagged far behind the solitary individuals.

Conclusions

In general the larger the number of snails grown in a confined aggregation the smaller will be the average size of the members of the association.

Isolated individuals of *Lymnaea palustris* grow faster and attain larger sizes than do individuals grown in an aggregation.

When growth is retarded by crowding during only the earlier period of the life cycle, recovery is usually complete after isolation. Continued crowding to a later time in the growth cycle produces a lasting effect from which full recovery does not follow. The result is a diminished final size of the snails.

Literature Cited

Allee, W. C., 1930. Animal aggregations. Univ. of Chicago Press.

Crabb, Edward D., 1927. The fertilization process in the snail, Lymnaea stagnalis appressa Say. *Biol. Bull.*, **53**: 67–108.

Crabb, Edward D., 1929. Growth of a pond snail, Lymnaea stagnalis appressa, as indicated by increase in shell-size. *Biol. Bull.*, **56**: 41–63.

Hammond, E. Cuyler, 1938, 1939. Biological effects of population density in lower organisms. *Quart. Rev. Biol.*, **13**: 421–438; **14**: 35–59.

Hoffman, H., 1927. Ronn's Klassen und Ordnungen des Tier-Reichs. *Physiologie Wachstum.*, III: Abt. 2, 1101.

ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1942

JULY 21

The effect of some vitamins of the B-complex on respiration of mutants of Neurospora. A. C. Giese and E. L. Tatum.

Using the standard Barcroft-Warburg technique the effect of the addition of various vitamins to cultures of Neurospora appropriately starved for the vitamin was determined. Addition of B_1 to a culture of a "thiaminless" mutant of Neurospora resulted in a small but significant rise in the rate of respiration.

Experiments were next performed with three vitamins of the B-complex-pantothenic acid, p-aminobenzoic acid and B_6 (pyridoxin) whose respiratory function, if any, has not yet been demonstrated. Addition of pantothenic acid to the "pantothenicless" mutant, of p-aminobenzoic acid to the "p-aminobenzoicless" mutant and of B_6 to the "pyridoxinless" mutant resulted in each case in a significant rise in the rate of respiration. This indicates that each of these vitamins has a respiratory function in Neurospora.

The increase in the rate of respiration is almost immediate in the case of B_1, somewhat slower in the other three cases suggesting that in the latter three the effect of the vitamins may be indirect but the experiments do not permit of a decision at present.

AUGUST 4

The effect of temperature on vertebral variations in Fundulus heteroclitus. M. L. Gabriel.

In spite of much study of temperature-controlled variation of vertebral numbers in wild populations of fishes, evidence from laboratory experiments is still needed to assure adequate control of temperature as well as genetic control otherwise impossible. In the present study, *Fundulus heteroclitus* eggs were fertilized *in vitro*, each stripping was divided into three parts, and the eggs were raised in constant temperature baths at 24.5° C., 18.7° C., and 13.5° C., the sibs being kept separate. The adult number of vertebrae is present at hatching and vertebral counts of alizarin preparations were made.

Significant differences in the mean vertebral number were obtained: the mean is $32.73 \pm .03$ (S.E.) at 24.5°, $33.10 \pm .04$ at 18.7°, and $33.44 \pm .11$ at 13.5° ($t = 3.12$, $P < .01$; $t = 6.44$, $P < .01$). At a single temperature (24.5°), the vertebral number was found to be related to rate of development. In fishes grouped according to time at hatching in two-day intervals, the mean rises linearly from $32.45 \pm .06$ at 9–10 days to $33.10 \pm .13$ at 19–20 days.

The hypothesis is advanced that the rates of somite separation and mesodermal histodifferentiation may be so related that under accelerating conditions the latter becomes precocious relative to somite formation so that vertebral rudiments may fail wholly or partly to separate. Fewer vertebrae would thus be formed, the modification taking place in the caudal region where the rate discrepancy would be greatest. It has been shown (Ford, 1933, *Jour. du Conseil*, **8**) that the caudal

region is the site of modifications in vertebral number. It is thought possible that "fused" or "complex" vertebrae, most common in the caudal region and at high temperatures, may be produced by such rate discrepancies between somite separation and histodifferentiation.

Some effects of temperature in the regeneration of Tubularia. Florence Moog.

In furtherance of recent work (Spiegelman, Goldin, and Moog) indicating that the determination of size of the hydranth in regenerating Tubularia is independent of the rate of formation to a limited degree, studies were made of the effects of temperature on the two factors. Temperatures of 7°, 10.8°, 13.5°, 18.7°, and 20.8° C. were used.

The relation between the velocity (the inverse of the time to the appearance of the constriction between the primordium and the rest of the stem) and temperature is linear, the steepness of the line varying directly with the rate at the highest temperature. The average Q_{10} was 1.8. The fact that the more sluggish material was least affected by temperature suggests that the velocity was being limited by low concentration of substrate.

The size of the primordium averaged 35 per cent longer at 7° than at 21°, the relation between size and temperature taking the form of a sigmoid curve. The average Q_{10} for the size effect was 1.29, and it did not vary much with size or speed. Apparently the determination of size is limited principally by a physical factor, probably diffusion. The change in size was too great to be accounted for on the basis of the increase in oxygen tension with decreasing temperature; more likely the slowing of the velocity constants of the processes of formation of the hydranth has the effect of raising the supply of substrate and oxygen available for conversion.

The effect of temperature change on the tentacle number is two-fold, the average number in the oral circlet increasing from 11.7 at 21° to 13.7 at 7°, while the basal circlet average decreased to 13.3 to 11.2. The ratio of the number in the oral to the number in the basal group shifted from 0.88 to 1.22, and the relationship between the numbers in the two circlets showed a significant correlation at each temperature, indicating that the processes determining the numbers in the two groups are interdependent. The data offer no basis for deciding whether the relative increase of activity of the oral portion of the primordium is an intensification of the normal dominance of the stem, or merely a local situation.

Mass and time relationships in the regeneration of Tubularia. S. Spiegelman.

Definitions of ratio of regeneration are necessarily arbitrary. Most early investigators were content to use $1/t$ as a measure of regeneration rate, where t was the time from section to a specified morphological stage in the process. This particular definition necessarily ignores the masses of tissue transformed, an important aspect of the rate problem. Accordingly Barth (*Physiol. Zool.*, **11**: 179, 1938) formulated a rate in terms of the ratio of the length of the primordium formed and the time required, i.e., L/t.

The mass of the primordium formed is proportional to $\pi r^2 L$, where r is its radius and L its length. Therefore the L in the definition is proportional to the mass of the regenerating tissue. It is thus seen that two distinct factors are combined in the ratio, one being the mass of tissue transformed into hydranth and the other, the time necessary to arrive at a particular morphological event in the process. It is clear that the rate of regeneration may be varied either by affecting separately the mass of tissue involved, or the time to constriction or both simul-

taneously. There is, therefore, no *a priori* reason for believing that regeneration rates modified by different reagents or even various concentrations of the same reagent are directly comparable since the same resultant rate could be obtained in several different ways involving entirely different mechanism. If this is true then similar effects on rates by two agents does not necessarily imply that they are acting through the same mechanism.

To test whether a separation of the mass and time factors is experimentally obtained experiments were carried out with various respiratory inhibitors. Two representative experiments are cited.

In the case of ethyl methane over a concentration range which yielded a 67 per cent decrease in the time factor, the mass factor was increased by 11 per cent. The resulting effect of course being a decrease in the L/t curve. In the case of cyanide within a concentration range which yielded over a 200 per cent decrease in the l/t factor, no significant change occurred in the mass.

It is possible to interpret these results in terms of a synthetic reaction requiring an energy source in a system approaching the steady state. However, whatever be the final interpretation of the data, it is obvious that under the circumstances the l/t definition of rate so commonly used is inadequate, and if the whole story is to be obtained both the mass and the time must be considered.

August 18

The contractile mechanism in unicellular chromatophores (melanophores of Fundulus). Douglas A. Marsland.

Contractility in the melanophore, whereby the pigment granules are withdrawn from the numerous branches and concentrated in the center of the cell, appears to depend upon a gelation of the protoplasm (plasmagel). As in the case of amoeboid movement, the streaming movements of plant cells and cleavage movements of egg cells, contractile properties develop in proportion to the degree of protoplasmic gelation.

Hydrostatic pressure in the range up to 8,000 lbs. per in.^{2} limits the gelation capacity in a fashion that parallels the inhibition of contraction. This is true not only in the steady contraction induced by KCl and adrenalin solutions, but also for the alternating contractions which appear when pulsation of the melanophores are induced by the Spaeth method. The pressure effect is the same regardless of whether or not there is a survival of the nerve fibres which normally initiate the expansion and contraction of these melanophores.

The contracting effects of KCl and adrenalin can be counteracted by reducing the temperature to 6° C., and the expanding effects of NaCl, acetylcholine and physostigmine solutions are cancelled at 32° C. To appreciate the significance of these results it must be remembered that all of the protoplasmic gels thus far studied display a behavior opposite to that of gelatin. All the protoplasmic gels have their counterpart in such gels as myosin and methylcellulose in which gelation is fostered by increasing temperature, and by decreasing pressure.

The stiffness of the protoplasmic gel in melanophores is considerably greater than in most other cells previously studied. However, with a centrifugal force of 80,000 × greater one can always demonstrate a displacement of the pigment granules in expanded specimens, but never in contracted ones.

Comparing the melanophore to an amoeboid cell,—the clear region which can be seen in the center of expanded and half-expanded specimens, probably represents the plasmasol, which is surrounded by the pigment-laden plasmagel. During the contraction of the plasmagel it appears as though the hyaline plasmasol is squeezed forth into the outlying branches of the cell, replacing the pigmented plasmagel as it retreats from these branches.

Aggregation of separate cells of Dictyostelium to form a multicellular body. Ernest H. Runyon.

The fruiting body of Dictyostelium is fungus-like, consisting of a multicellular stalk anchored to the substratum, and a terminal mass of spores; but each cell of this structure was previously an independent protozoan-like amoeba (myxamoeba). Thousands of amoebae, after a period of feeding and multiplication come together in characteristic patterns forming a pseudoplasmodium, in which there are no cell fusions, but nevertheless as has been well demonstrated by Raper, definite co-ordination and dominance. The pseudoplasmodium is the immediate precursor of the fruiting body.

Study of the aggregation phase was found to be facilitated by the use of non-nutrient agar over which are thinly spread the amoebae, previously washed, concentrated, and roughly separated from the associated mass of bacteria by centrifuging. Size, pattern, and rate of development of aggregates are much affected by the thickness of the film or layer of water in which the amoebae are dispersed. If the culture is relatively dry (the aqueous film very thin) collecting points (centers) are many and aggregation progresses rapidly. In thicker films aggregation is slower, centers fewer, and the amoebal strands leading to the centers longer and less compact. Under these conditions, also, spiral aggregation patterns are frequent. Under water 0.2 to 0.5 mm. deep the aggregation pattern is a network of amoebal clumps connected by much elongated but loosely placed amoebae; there are no well delimited centers nor compact strands. The aggregation influence seems to be diffuse. Under water deeper than 1 mm. aggregation has not been observed, but the relatively small proportion of the amoebae that comes into the surface film does aggregate there. Potts in 1902 recorded fruition of Dictyostelium under oil. The pattern and rate of aggregation under a layer of paraffin oil is much the same as without the oil: the collecting points are many and aggregation progresses rapidly. Aggregation with center determination occurs under a glass cover slip or dialyzing membrane (Visking) placed over the amoebae on agar. Amoebae which are on top of such a dialyzing membrane become oriented corresponding to patterns of aggregation below the membrane: the amoebae on top accumulate along strands and centers below the membrane. Thus it seems likely that the determination of centers of aggregation depends upon the diffusion of a substance that is water- but not oil-soluble, active in thin films, and of a molecular character such that it can pass through a dialyzing membrane.

The structure of biologically active membranes. Dorothy Wrinch.

The membranes under consideration are plasma membranes, flexible and highly specific systems containing a protein component. Plainly we must assume that the proteins are in their native state, since only then is high specificity evinced. Further the membranes must be continuous with the cytoskeletons within the cell, so that theories attributing to the membranes structures of radically different types from those of protoplasm are necessarily excluded. Actually, extending to the membranes the type of structure already proposed for the cytoskeletons, on the basis of the newer knowledge of native protein structure (Wrinch, *Cold Spring Harbor Symp.*, 1941), the required properties result.

Thus native proteins are characterized by their ability to form associations. On this view the membranes consist of native proteins linked to form a surface skin, continuous with the interior cytoskeletons. Interlinks are of two types, less polar links where the hydrocarbon groups cushion together and more polar hydrogen bridges or ion rivets which are known to impose a rather open structure showing that our membrane should have pores. The passage of molecules will be distinctively different in the two cases. Growth, proceeding by the incorporation of new molecules, must exhibit the highest specificity and this "activity" the most

characteristic feature of all living systems may be studied as an expression of the high and specific associability of native proteins.

This picture of membranes as surface networks of native proteins suggests a new line of experimental attack, in which *the native protein* is the central theme. This theme cannot be set aside lightly, for the proteins are ubiquitous and their structure-determining powers in native form are approached by few other molecular species and rivalled by none. Given a fuller extension of Langmuir's powerful techniques, there is no apparent reason why these experiments should not be extended to yield information as to the formation of mixed films of native proteins and other biologically significant ions and molecules.

August 25

Experimental modification of molts and coat-color-changes by controlled lighting of the short-tailed weasel. Thomas Hume Bissonnette and Earl Elmore Bailey.[1]

For over three years, two short-tailed weasels (*Mustela cicognanii cicognanii* Bonaparte), similarly fed and cared for, under temperatures 40–70° F., were subjected alternately to controlled daily periods of available light. Both maintained normal molts and color cycles on normal light-cycles. Normal pelt-cycles were resumed following return to normal days after experimental light-manipulations. Molting, followed by growth of more or less white pelt, followed shortening daily light periods, even in March–April, when control, in the same room, on normal days turned brown. While experimentally lighted, one passed a summer in piebald coat, after incomplete color-change in both directions, and in almost complete white the next summer on normal days, followed by normal autumn molt to complete white and spring molt to nearly complete brown coat. One whitened in October, on short days, and returned to brown in December, on long days. The other responded likewise under similar light-manipulation. Gradually or suddenly shortened days induced whitening; lengthening days, brown, regardless of previous color. Molting was stopped short of completion by appropriate lengthening or shortening of daily periods of available light. Temperature-changes did not condition these differences. Changes in daily lighting constituted the major factor inducing and controlling these molts and color-changes in these Pennsylvania-bred weasels, studied in Connecticut.

Thiamin (vitamin B_1) deficiency in the cat. Sixteen millimeter film. Guy M. Everett and Dietrich C. Smith.

A sixteen millimeter motion picture film was made showing the behavior of two cats during the development of thiamin deficiency. After three weeks on the vitamin B_1 deficient diet, during which time the two cats lost approximately 20 per cent of their body weight, the animals showed muscular weakness and marked disturbances in postural tone. Walking was awkward and unsteady. The righting reactions were much impaired and pupillary constriction in bright light was lessened. Interest in food had ceased. At this time any slight stimulus may have produced a convulsive seizure lasting several minutes. Both clonic and spastic spasms occurred.

Upon the injection of one milligram (333 units) of thiamin chloride the animals showed within an hour improved muscular coordination and a return of

[1] Aided by grants from the Penrose Fund of the American Philosophical Society, 1939, and the American Academy of Arts and Sciences, 1942.

appetite. Convulsive seizures could no longer be evoked. In twenty-four hours recovery was practically complete.

Papers by Title

Permeability of Pelomyxa carolinensis to water. W. H. Belda.

Pelomyxa carolinensis (Wilson) is a multinucleate amoeboid organism of relatively large size. The changes in volume which occur when specimens are kept in solutions of different osmotic concentration can readily be measured.

A culture of Pelomyxa was obtained from the General Biological Supply House, Inc., which designates this organism *Chaos chaos,* Schaeffer, Strain A. The specimens were grown in Hahnert solution in glass finger bowls and were fed on *Stentor coeruleus.* At intervals, large specimens of Pelomyxa were put into fresh Hahnert solution for 48 hours, until all food vacuoles had disappeared, and the volume of each was measured by means of a volumescope. This apparatus consists of a glass tube having a bore about 0.25 mm. in diameter. When a specimen of Pelomyxa is drawn by suction into the tube the organism becomes cylindrical in shape. Its volume is then directly proportional to its length.

Specimens kept in Hahnert solution without food showed an average decrease in volume of 8% every 24 hours.

Specimens transferred to 0.1 M., 0.2 M., and 0.3 M. solutions of non-electrolytes (mannitol, erythritol, and lactose) in Hahnert solution, and to distilled water underwent various changes in volume, as shown by the following table. Each quantity represents the average volume of 50 or more specimens in terms of per cent of the original volume in Hahnert solution.

Medium	Time in hours after transfer					
	1	2	4	6	12	24
0.1 M. non-electrolytes	92.7	91.1	84.6	80.3	74.5	69.3
0.2 M. non-electrolytes	82.6	73.2	59.4	51.9	29.9	33.2
0.3 M. non-electrolytes	78.1	63.8	disintegrated			
Distilled water	108.2	102.5	103.9	97.5	99.6	

After having been kept in 0.1 M. solutions of non-electrolytes for periods of from one to 12 hours, the specimens usually recovered their original volume if they were returned to Hahnert solution for an equal period of time. Specimens which had undergone shrinking in higher concentrations did not recover their original volume completely.

The rate of output of fluid from the contractile vacuoles of Pelomyxa was measured. Specimens kept in Hahnert solution eliminated 3.8 per cent of their total volume per hour. In distilled water the rate of output was 6.4 per cent of the total volume of the organism per hour. After transfer from Hahnert solution to 0.1 M. solutions of non-electrolytes the output of fluid from the contractile vacuoles decreased rapidly to nearly zero within one hour.

The value of k, the permeability constant for water in *Pelomyxa carolinensis,* was calculated. Under various conditions of swelling and shrinking k was usually between 0.02 and 0.03 cubic micra of water per square micron of surface per minute for each atmosphere of pressure.

The effect of thiamin chloride on the oxygen consumption and the development of Arbacia punctulata at different stages. Matilda Moldenhauer Brooks.

Experiments on the rate of O_2 consumption of different stages in the development of Arbacia as affected by different concentrations of thiamin chloride were done using the Warburg-Barcroft method. Fourteen concentrations were used from 2×10^{-4} to 2×10^{-11} and from 4×10^{-5} to 4×10^{-8}, diminishing in value by the factor of 10. The stages used were unfertilized eggs, fertilized eggs, early cleavages, morula, blastula, gastrula and pluteus at a temperature of 15.9° C. There was no change in the rate of O_2 consumption in any of these experiments during three to four hours in which the experiments were conducted.

The rate of development appears to have been affected. Thiamin chloride, in concentrations of 1×10^{-7} and 10^{-8} was added as follows to eggs in sea water: (A), immediately before fertilization; (B), immediately after; (C), at the 2 to 4 cell stage; (D), the morula; (E), the blastula; (F), the gastrula; (G), the pluteus. All experiments were done under similar conditions as to egg suspension, volume of solution, temperature (running sea water.) At the end of 24 hours (A) contained 50% gastrula and 50% pluteus, while the controls contained 95% gastrula and 5% pluteus; in (B) there were 100% pluteus; in (C), 100% pluteus. The size of the pluteus from the tip of the spine to the end of the larva was measured, in (A) to (F). These showed a length of .35 mm. in 3 days as compared with .27 mm. in the controls. They remained larger for a longer time than the controls and lasted 14 days in (G) as compared with 6 days (controls).

These experiments show that there is no direct relation between the utilization of thiamin or the rate of development, and the O_2 consumption; there appears to be a direct relation between the intake of thiamin and the rate of development and length of life especially marked in the early stages and the pluteus.

It would appear that the oxidation end of the redox scale in metabolism is not directly affected by thiamin chloride.

Intake and loss of phosphate ion by eggs and larvae of Arbacia and Asterias. S. C. Brooks.

This material was immersed in and sampled from a beaker, or each sample taken from a separate dish, or flooded in a Buchner funnel, using radioactive sodium phosphate in low concentrations (0.175–0.81 mM. L^{-1}). The samples were separated by washing with isotonic erythritol (which removed much of the radioactivity), or simply by hard centrifugation, and the phosphate content of eggs determined by counts. The radioactivity as measured with a counter was translated into concentration of the phosphate ion.

It was found that phosphate had often been absorbed in two distinct periods separated by a period of loss of this ion. Assuming arbitrarily that the concentration was the driving force during intake, it is found that the early rate indicates a permeability for Arbacia of $36\text{–}70 \times 10^{-10}$ and for Asterias 2280×10^{-10} GM. $cm.^2$ $hr.^{-1}$ $(GM.L^{-1})^{-1}$. For the late intake, values of $2.2\text{–}70 \times 10^{-10}$ were found. The maximum concentrations found in the eggs or larvae of both vary between 0.0050 and 8.3 mM. L^{-1}. Inverse correlation was intimated by the data between concentration or radioactivity and permeability. Tests of intake of radioactive sodium were done. Full publication will discuss the effects of the β-radiation (the sole radiation of P^{32}), the part played by the stage of the egg or larvae, the effects of the methods used, the dimension of different ions and theories of absorption.

Oxygen consumption in caffeinized Arbacia eggs. Ralph H. Cheney.

As a fundamental step in a general investigation regarding the effects of caffeine upon the reproduction and growth of animals, experiments were conducted

to determine the action of caffeine directly upon the micro-respiration (in terms of oxygen consumption) of the fertilized eggs of *Arbacia punctulata*. Mature eggs were fertilized and prepared in the form of a 35 per cent suspension in sea water. The following percentages of caffeine-in-sea-water were employed: 2, 0.5, 0.2, 0.1, 0.02, 0.004 and 0.002. Five-tenths of one cubic centimeter of the 35 per cent suspension of fertilized eggs was used in all experiments. Sea water or the proper percentage of caffeine-in-sea-water was added to Warburg flasks so that a total volume of 2 cc. of fluid was maintained in all vessels. Experiments were conducted at 25° C. The determination of the oxygen consumption per hour for a total of three hours was made by means of the Warburg manometers and method.

Total oxygen consumption of controls and the experimental series was plotted as a time relationship curve in cu. mm. of oxygen consumed over a three hour period. Variations from the untreated controls were from 0.5 to 61 per cent. Changes of less than 10 per cent, whether inhibitory or stimulatory, were considered not significant in biological material of this sort. The oxygen consumption of fertilized eggs of Arbacia was inhibited by concentrations equivalent to 0.1 per cent or above. In the series employed, lower percentages caused a variation of 10 per cent or less and were interpreted as not significant.

A typical experiment (number 20 of the series) inhibited the oxygen consumption over a three hour period statistically as follows: 2 per cent caffeine-in-sea-water reduced oxygen consumption 60.6 per cent in comparison with controls, 0.5—58.6 per cent, 0.2—41.4 per cent, 0.1—22.9 per cent reduction. Eggs were examined with 100 × magnification after each experiment to check the cleavage stage in each vessel against the controls and against the corresponding percentages in other parallel experiments of the series.

Caffeine effect on fertilization and development of Arbacia eggs. Ralph H. Cheney.

To determine the extent of the effect of caffeine upon the ability of the egg to become fertilized and of the sperm to accomplish fertilization, eggs and sperm were shed separately into caffeine-in-sea-water and into normal sea water. The germ cells could then be mixed as desired in the various combinations available. The production of the fertilization membrane and subsequent cleavage stages developing in time periods comparable to the normal controls were studied. Normal time periods for the different developmental stages were accepted as presented by E. B. Harvey (*Biol. Bull.*, **79** (1): Plate II, photographs 16–32 inclusive, August, 1940). The following percentages of caffeine-in-sea-water were employed: 0.00, 0.002, 0.004, 0.02, 0.04, 0.1, 0.2, 0.5, 1.0 and 2.0. Eggs and sperm were shed into the normal sea water or into each of the above concentrations separately. They remained in the solution in which they were shed for fifteen minutes prior to mixing in any combination.

For each percentage used in the series, the combinations of normal and caffeinized eggs and sperm were mixed as indicated below:

1. Normal non-treated egg × normal non-treated sperm and development allowed to occur in normal sea water. These served as controls for each day's run.
2. Normal egg × caffeine-treated sperm. Development in sea water.
3. Caffeinized egg × normal sperm. Development in sea water.
4. Caffeinized egg × normal sperm. Development in sea water—caffeine.
5. Caffeinized egg × caffeinized sperm. Development in sea-water.
6. Caffeinized egg × caffeinized sperm. Development in sea-water—caffeine.

Experiments were conducted at 25° C. An examination of the eggs was made at intervals of 10, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180 minutes and at longer periods up to two to three days or until either pluteus larvae developed or cytolysis occurred.

Plutei developed in normal *time* and *form* in all combinations of 0.002 per cent and 0.004 per cent caffeine-in-sea-water. All combinations except the caffeinized egg × caffeinized sperm developed in caffeine-in-sea-water in 0.02 per cent and 0.04 per cent, developed plutei of normal form but in delayed time. In 0.1 per cent and 0.2 per cent cleavage was arrested at various stages and the echinochrome pigment became localized. Higher percentages affected the egg so that the cleavages became progressively more abnormal and the arrest of division occurred at an increasingly earlier stage. Two percentum prevented the formation of a fertilization membrane in combinations numbers four and six. There were many evidences that the egg was more sensitive than the sperm to the action of caffeine.

Effects of irradiated water on Arbacia sperm. Titus C. Evans.

In previous investigations on the influence of the medium on the radiosensitivity of Arbacia sperm, check experiments using irradiated water showed no effect on unirradiated sperm. Recent attempts have been made to determine whether slight amounts of a toxic agent produced in sea water irradiated with very large doses of x-rays could be detected. Positive results have been obtained with doses of the order of 100,000 roentgens; using very dilute concentrations of sperm (1:4000); and allowing the sperm to stay in the irradiated water for a sufficient length of time. The irradiated sea water under these conditions caused the sperm placed therein to die sooner than the control sperm in sea water. In addition, it was found that sperm kept in irradiated sea water until approximately 50 per cent had died, caused a delay of about 10 minutes (at 22° C.) in the time of cell division of eggs fertilized therewith.

The deleterious agent produced by the radiation in the sea water is apparently hydrogen peroxide. Tests with titanic chloride reveal a very faint color change in heavily irradiated sea water which roughly corresponds to a concentration of hydrogen peroxide between 1:1,000,000 and 1:2,000,000. Hydrogen peroxide in a concentration of 1:1,000,000 affected the sperm in much the same way as did the irradiated water.

Protein metabolism and embryonic growth rates. Otto Glaser.

Nitrogen in chicks (Glaser) supports Schoenheimer's conclusion that protein metabolism in growing organisms and adults differs mainly in the ratio synthesis/destruction. Temporary storage of blood proteins or the capacity to retain more than usual quantities of particular amino-acids (van Slyke: Riker) fail to demonstrate storage of amino-acids unless as substituents in existing proteins or as building blocks specifically located in new molecules.

With certain native proteins immediately or indirectly responsible for all other accumulations and syntheses, the only amino-acids that control our measurements are the ones that settle in appropriate locations. Granted adequate income, the number of amino-acid units that settle during a given time equals the number of available locations. During development, progressive cell differentiation reduces the rate at which new locations are produced. Hence the rate of amino-acid settlement should decline by steps proportional to differences between the squares of consecutive whole numbers (Glaser).

This fact, usually obscured by heterogonic diversifications, leaves its imprint on properly timed growth rates. In the linear equation

$$\log w = k \log(2t + 1) + C,$$

C represents a weight at zero time, w a weight $2t + 1$ conventional time units later and k, the rate. Here the time-scale has intervals systematically proportional to differences between the squares of consecutive whole numbers. By this scale k for the entire chick equals k for its genetic proteins. For isolable organs and native

chemical fractions, the k's have ratios identical with the corresponding allometric constants. The only processes to which this numerical system can plausibly apply, are protein maintenance and synthesis. Moreover, a metrical system so specifically implicating surfaces would be exceedingly unlikely unless the native protein molecule is organized as a compact three-dimensional structure, carrying its many specificities on a well-defined surface.

Rate of breaking and size of the "halves" of the Arbacia punctulata egg when centrifuged in hypo- and hypertonic sea water. Ethel Browne Harvey.

Arbacia eggs kept in hypotonic (60 per cent, 80 per cent) sea water break less rapidly and those kept in hypertonic (125 per cent) sea water break more rapidly than those in normal sea water when centrifuged in sugar solutions of corresponding tonicities at 10,000 × g. The break occurs so that the red (granular) "half" is practically the same size in all the solutions, and the increase (in hypotonic) and decrease (in hypertonic) in size is in the white (clear) "half."

Some observations with a simplified quartz microscope. George I. Lavin and Arthur W. Pollister.

A quartz microscope has been assembled for work with the mercury resonance line 2537 Å. The light source is a quartz mercury whose output is about 90% 2537 Å. The visible light is removed by means of a liquid filter (mixture of $CoSO_4$ and $NiSO_4$) described by Bäckström, 1940 (*Arkiv. for Kemi. Mineralogi Och Geologi,* 13: 1). In operation an image of the material to be examined is focussed on a synthetic willemite screen which is fastened, face down, to the usual ground glass holder. It has been found that when the image is in focus on this screen it is in focus when a plate is taken with the 2537 Å line as the light source. This line is useful in biological work because it is close to the region of maximum absorption of the purine and pyrimidine components of nucleic acid. Photographs of fibers of nucleohistone show intense absorption. Photographs of liver cells that have been treated by a method known to remove thymus nucleohistone shows that the nuclei have lost practically all of their nucleic acid, since they absorb very slightly. Living spermatids of insects have been photographed. The mitochondrial body or nebenkern (a sphere 20 micra in diameter in *Notonecta undulata*) absorbs no more intensely than does the surrounding cytoplasm; so the indications are that the mitochondria are poor in nucleic acid. The Golgi bodies (acroblasts) show appreciably more absorption than the general cytoplasm.

Pituitary function in the chromatic physiology of Opsanus tau. Richard E. Lee.

The melanophore and xanthophore pigments of the toad-fish, *Opsanus tau,* become dispersed over a black background and assume a partially concentrated condition over a white background. The chromatophores of denervated bands behave similarly under these conditions, but their pigment migration is much slower and less extensive than that found in the innervated cells.

Hypophysectomy has no apparent effect on this ability of toad-fish to change color. Likewise, denervated chromatophores of hypophysectomized specimens will repeatedly disperse their pigments after having assumed a concentrated state in response to white backgrounds or to electrical stimulation of the denervated region. The ventral integument of hypophysectomized animals, however, contains approximately 50 per cent of the usual number of melanophores; and 20 to 30 per cent of these remaining cells generally show signs of degranulation and melanin loss.

Biological assay of toad-fish pituitaries reveal that the average gland contains 525 frog units of melanophore dispersing principle. At least 1300 frog units of this medium (obtained from toad-fish pituitary glands or commercial pituitrin) are necessary to produce a slight transitory dispersion of all toad-fish chromatophores within a radius of 0.5 cm. about the point of a subcutaneous injection.

It is concluded that the toad-fish pituitary gland functions to sustain coloration by pigment maintenance, but it has no apparent role in the more overt "physiological" color changes of this animal.

The interfibrillar material in the central nervous system of mosquito larvae (Culex pipiens). A. Glenn Richards, Jr.

Sudan dyes used as indicators of the distribution of oil solvents (e.g., xylol) via the tracheal system give bright coloration to only the central nervous system. The concentration of dye agrees with the known concentration of intercellular and interfibrillar material. Ganglia just beginning to be stained by the above method show that the dye diffuses from the small tracheae and then along the fibers. These data suggest an interneuronal lipoid but these nerves are not medullated since they do not reduce osmic acid except after heat fixation. After formalin or acetic acid fixation this lipoid is stained as a non-resolvable diffuse color along fiber tracts and between cells, especially with Black Sudan B. It can be similarly stained by brief immersion of living ganglia in the alcoholic stain but alcohol soon releases Sudan-stainable particles (mostly $< 10\,\mu$) which are readily removed by strong alcohol and slowly by weak alcohol or water. These particles have irregular shapes, a high melting point ($> 100°$ C.) and negative birefringence. Clearly they are different from the lipoids of adipose tissue. This substance seems to be a bound phospholipid.

Data published in a previous paper show histological degeneration of the fiber tracts of the central nervous system due to toxic petroleum oils. One of the effects of such oils seems to be the destruction of this interfibrillar material. It is suggested that this material permits the rapid penetration of oils and oil-borne toxins into the central nervous system. If this substance is a phospholipid its breakdown may under some circumstances produce degeneration products which are neurotoxins.

A fourteen-day rhythm in the left-right spiraling ratio of the common marine ameba Flabellula citata. A. A. Schaeffer.

In common with other species of amebas and human leucocytes (polymorphs), the common marine ameba moves around a capillary glass tube in a helical spiral path. A part of the path is twisted in a right spiral and a part in a left. The amount of the path to the left as compared with that to the right is the left-right ratio.

This summer daily records of the left-right ratios of Flabellula from a pure line culture (27.7° C.) were obtained. When these ratios are plotted vertically on cross section paper with the days as a horizontal time scale, pronounced 14-day rhythms are observed in the ratios. These rhythms sweep diagonally across the graph from the left to the right. Each rhythm takes about 14 or more days for completion. For the greater part of the time two rhythms are present at the same time. The ratio of the amebas in any one rhythm become more right as time goes on, at the rate of .1 each day. That is, if the ratio is 1.7 left to 1 right on July 24, then on July 25 the ratio will be 1.6 : 1. When this rhythm reaches a ratio of about 1 : 2 right, it stops and a new rhythm appears with a predominant left ratio. Thus, while the rhythm from left preponderance to right preponderance is steady and continuous, there is visible no return rhythm from right to the new left rhythm. That is, the rhythms are discontinuous as represented on the graph.

The presence of a 14-day rhythm in this ameba is of great interest, for two years ago a 14-day rhythm was found in human leucocytes at normal temperature (37.6° C.). This and similar earlier researches are guided by the hypothesis that the chief controlling mechanism consists of two stereoisomers of which the left one is more labile and breaks down at a constant percentage rate.

The early embryonic development of Amaroecium constellatum. Sister Florence Marie Scott.

Amaroecium constellatum produces heavily yolk-laden eggs that develop to the free-swimming stage within brood spaces in the adult. The first cleavage divides the egg into a smaller left blastomere and a larger right blastomere. This disparity in size of cells from left to right continues through subsequent divisions. The second cleavage separates two larger anterior cells from two smaller posterior cells. The third cleavage produces four micromeres at the animal pole and two heavy macromeres at the vegetative pole.

In the sixteen cell stage the embryonic areas may be distinguished. They correspond to the areas of the less heavily yolked Protochordates, Styela and Amphioxus, consisting of an anterior chorda-neural and a posterior mesodermal crescent enclosing an endodermal area. The areas are delineated at the animal pole, the vegetative hemisphere being composed of the yolk macromeres.

Gastrulation is initiated at the 22-cell stage. Divisions on the right side lag behind those on the left. The smaller size of cells and the accelerated rate of division of the mesodermal cells on the left concur to facilitate invagination on that side whereas the mass of yolk and slowed rate of cleavage interfere with ready invagination of mesoderm at the ventral and right lateral lips. The chorda-neural cells divide into their derivatives, the chordal cells turning in between the endoderm and the neural plate. The endodermal cells change in shape and they become slightly depressed but there is no invagination. By the approximation of neural cells and the ectoblastic derivatives of the ventral lip the blastopore closes.

The neural cells reacting, possibly, to the organizing influence of the leftwardly directed mesoderm grow down on the left side of the embryo. The neural plate thus curves through an angle of 90°. The dorsal region differentiates into the brain vesicle and ganglion, the lateral part into the neural tube. The neural tube lies to the left of the notochord which grows directly backward from the ventral lip differentiating partly from chordal cells and partly from cells enclosed by the process of overgrowth. The first mesoderm to invaginate at the lateral lips becomes mesenchyme. The remaining mesoderm is converted into muscle cells of the tail.

Endoderm, lacking an archenteron, is established definitely by the process of overgrowth, mesoderm by the process of invagination. The asymmetry of the nervous system obtains from interference with invagination at the ventral lip. The pattern of differentiation in Amaroecium in the same as that of the less heavily yolked Protochordates but the yolk introduces modifications of the plan that alter gastrulation by interfering with the convergence of morphogenetic substances toward the median axial plane.